Instituut voor Tropische Geneeskunde
Institut de Médecine Tropicale
Institute of Tropical Medicine
Instituto de Medicina Tropical

Nationalestraat, 155
B – 2000 Antwerpen

Stichting van Openbaar Nut 0410.057.701

POSTGRADUATE IN TROPICAL MEDICINE AND INTERNATIONAL HEALTH
MODULE 1 & 2
CLINICAL & BIOMEDICAL SCIENCES OF TROPICAL DISEASES

LABORATORY NOTES

Parasitology
Bacteriology
Hematology

SEPTEMBER 2018

Idzi Potters – Anali Conesa Botella – Jan Jacobs

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Human PARASITOLOGY in Tropical Settings

Practical notes

Idzi Potters - Philippe Gillet - Jan Jacobs

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Some preliminary remarks

About the taxonomy used in this syllabus:

Every species belongs to a Genus. This last one has a certain place in successively: a family, an order, a class and a phylum. The names of the classes and families, used in this syllabus, are not always those recommended by the International Zoological Nomenclature (Classification of the Committee of Nomenclature, Levine et al., 1980), they are however commonly encountered in parasitolgy. This simplified classification is used intentionally in an effort to avoid unnecessary complication.

Helminths of medical importance can be divided in two sub-groups:

- The phylum of Plathelminths (flat worms with rudimentary digestive tracts and a thin surface/skin through which a substantial part of the food is absorbed) comprises two classes that will be discussed in this syllabus:
  - The Trematodes: Non-segmented body and hermaphroditic (except for the schistosomes)
  - The Cestodes: Segmented body and hermaphroditic (every segment is bisexual)
- The phylum of Nemathelminths (round, cylindrical worms, non-segmented with complete digestive tract, separated sexes and with a tough, solid surface/skin) has only one important class:
  - The Nematodes

For the Protozoans (single-celled parasites), a simplified classification is suggested in this syllabus, mainly based on their type of movement, making it more useful in the laboratory diagnosis:

- The group of Amoebae: movement by pushing out the ectoplasm to form pseudopodia. Parasitic forms are always extra-cellular.
- The group of Flagellates: movement by means of one or more flagella, which are however not always present in the different evolutionary stages of the parasite.
- The group of Ciliata: movement and ‘capturing’ of food by means of co-operation of numerous cilia.
- The group of Sporozoa: no apparent way of moving. Parasitic forms are usually intracellular.

For some parasites no clear or unique classification is known at this time.

In this syllabus some bacteria and fungi are included, as their method of detection is the same as the ones already mentioned in these notes.

About the dimensions, given in this syllabus:

The dimensions of the parasites, given in this syllabus, are those, cited as the minimum and maximum dimensions by the World Health-Organisation (WHO) and/or the Centers for Disease Control and Prevention (CDC). In summarizing tables and schemes in this syllabus, the most commonly encountered dimensions will be given.

Idzi Potters
Philippe Gillet
Jan Jacobs

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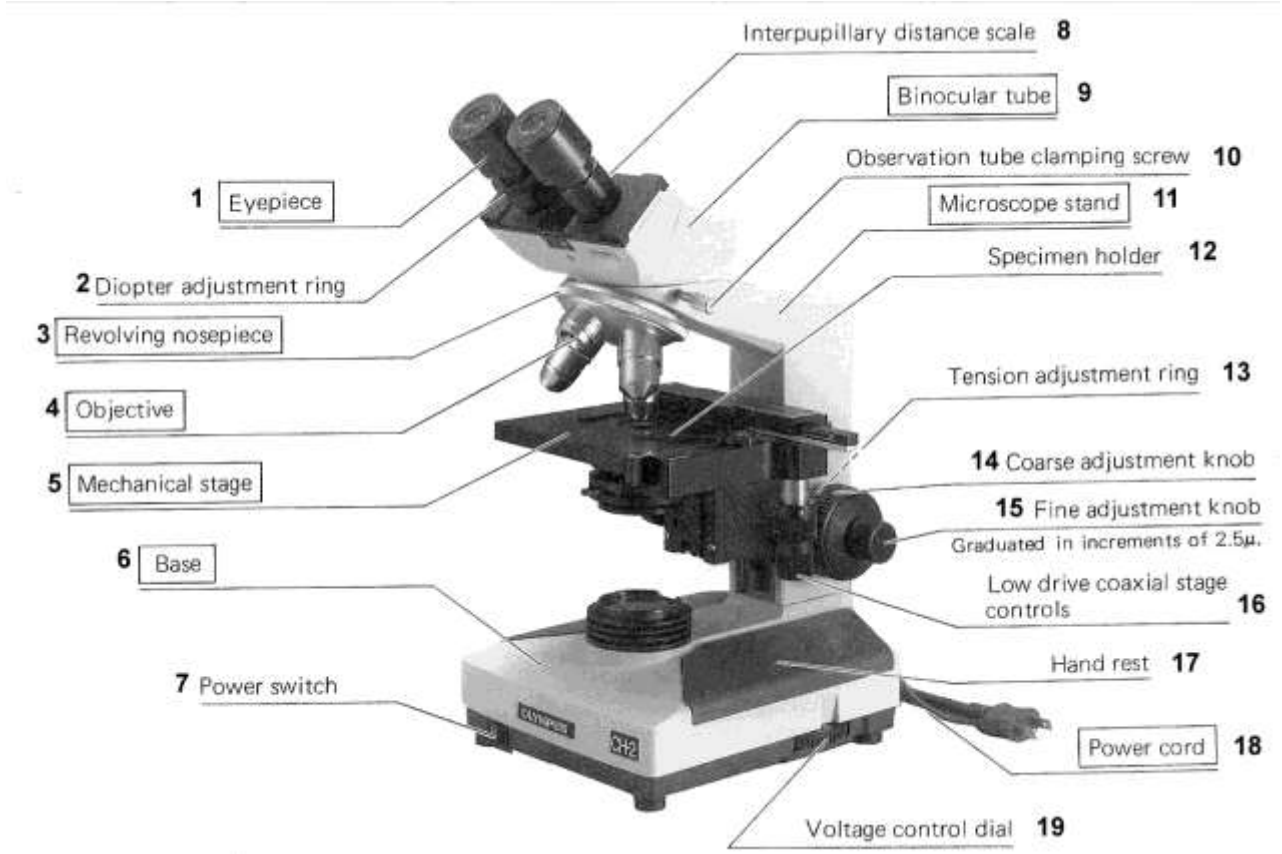
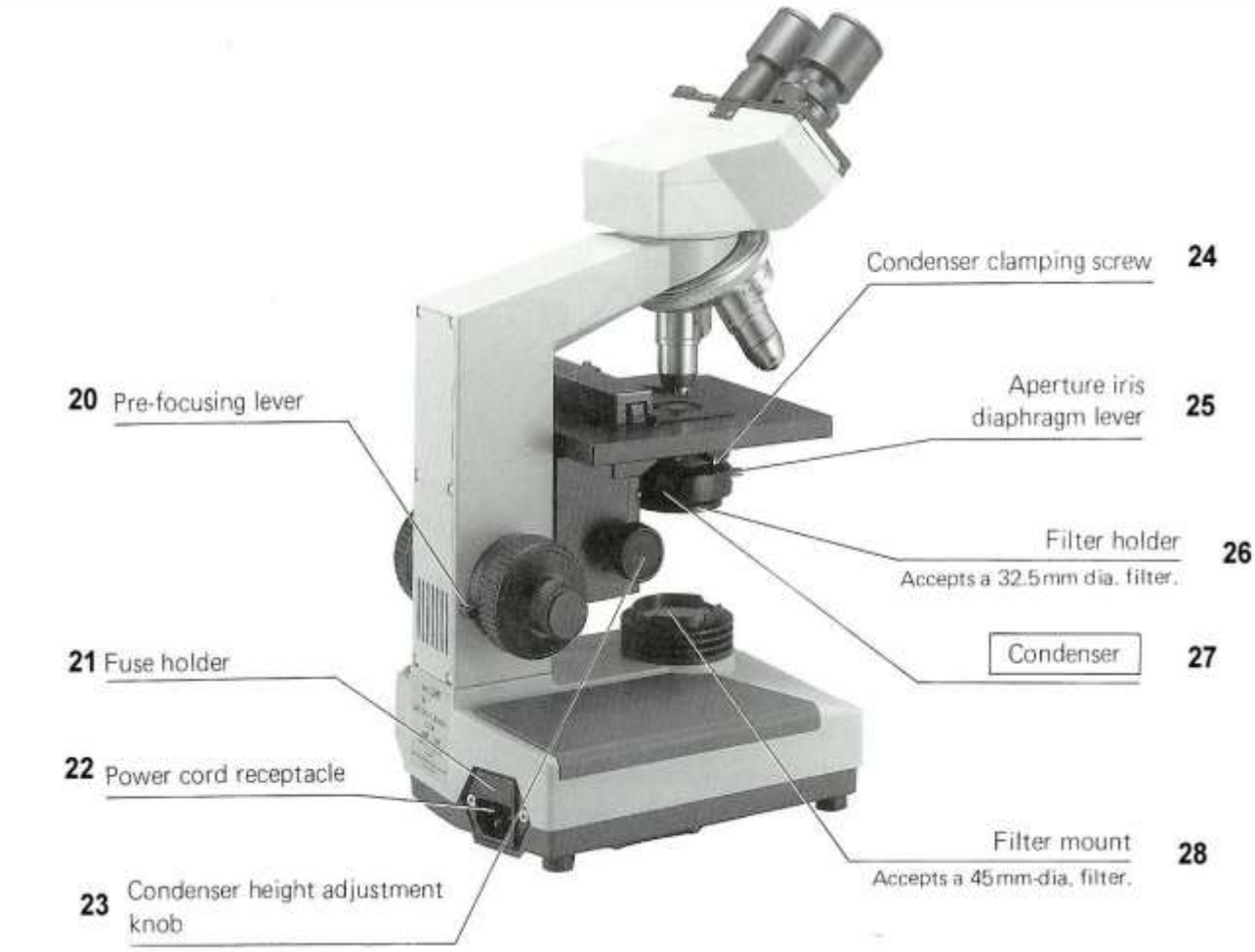
Description of the microscope

Used example: OLYMPUS, model CHK





Maintenance of the microscope

Already in the 17th century Antoni van Leeuwenhoek (1632-1723), a dutch biologist, developed the first, though rudimentary, microscope. Throughout the centuries different investigators have further expanded this device to the rather complicated tool that we know nowadays. The microscope consists of a series of extremely precise mechanical parts and extremely sensitive optics. For every microscope one should always check the enclosed manual for ideal transporting conditions and maintenance-techniques.
In isolated labs, the microscope is probably the most important tool one can find, for it can diagnose a whole set of bacterial infections (tuberculosis, meningitis,...) and parasitic infestations (trypanosomiasis, malaria,...). Even for other diagnostics, the microscope can give us useful surplus information (e.g. a count and/or identification of certain cell-types in biological samples).
Nevertheless the microscope remains a relatively expensive device which deserves, in other words, all our attention.

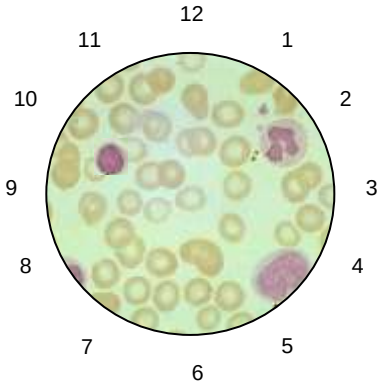
In a small laboratory, the microscope is an indispensable tool that should meet the highest standards. A poorly maintained microscope will drastically lower the quality and sensitivity of the tests. A microscope with fungal growth in the optical parts, which is not rare in moist and tropical conditions, is definitively out of order. For these reasons, the microscope should be handled and maintained with lots of care, so its lifespan can be prolonged for several years.

- Always avoid shocks. When transporting the microscope, a box or crate, especially designed for this purpose, should be used, in which the microscope is well immobilised.
- The microscope should be well-protected against dust. This is especially the case during the dry and warm season. Dust and sand-storms can seriously damage the mechanical parts of your microscope.
- In the warm and moist season fungal growth on lenses and prisms of the optical system poses the biggest problem.
- Certain precautions can be taken to avoid the problems mentioned above:
    - When using electrical microscopes, the intensity of the light-source should be set to the minimum intensity before switching off the microscope. This will substantially prolong the logevity of the light-bulb.
    - Always make sure that some spare lightbulbs for the microscope are in stock. Always keep the reference-numbers of all parts and accessories for replacing them if necessary.
    - One should remove the immersion-oil from the immersion-lenses on a daily basis by wiping it off with a non-fluffy, soft and clean tissue or with toilet-paper. Dried oil will cause the microscopic image to be foggy. When dried oil is sticking to the immersion-lenses, it can be removed, using a clean tissue, moistened with xylol or with a mix of ether and alcohol (1/1 v/v). This way of cleaning should be kept to a minimum and should never be used on internal lenses and prisms. After cleaning, the lenses should be dried immediately with another clean and dry tissue or toilet-paper. Also plastic lenses exist, which are not resistant to organic solvents. For this reason, one should always carefully check the manual of the microscope for suitable solvents.
    - Other lenses should be cleaned with another tissue, which is not used for the immersion-lenses, as any contact between oil and non-immersion-lenses should be avoided.
    - Never leave the tube of the microscope open (i.e without eyepieces) as this can cause dust and fungal spores to enter into the micrcoscope.
    - After every use, the microscope should be covered with a preferably cotton dust-sheet. Avoid plastic covers, as these will promote fungal growth, especially in moist circumstances.
    - The best thing to do is to place the microscope in an acclimatized room, but only when the acclimatization is never interrupted (danger of condensation within the optics of the microscope in case of fluctuating temperatures). At any rate, the acclimatization should never be set to a relatively low temperature. So, the temperature-shock in case of electrical break-down is not too big.
    - Local air-humidity can be diminished by placing an electrical dehumidifier, or even more simply, by placing dried silicagel-crystals (with saturation-indicator), near the microscope, in a closed cupboard.
    - After using the microscope one can also put it in a place, in which air-humidity is diminished by means of a low-power light bulb (± 20 Watt).

The microscopic field

The **microscopic field** is the circular image one sees at a certain magnification.

This microscopic field can be considered to be the plate of a clock. Doing so, we can always locate any object in this microscopic field, starting from the centre:

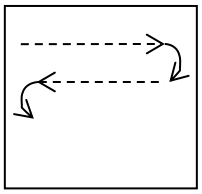


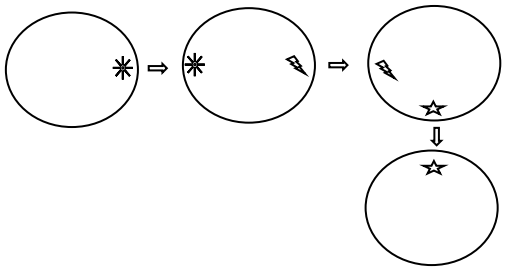
Examples:

- Between one and two o'clock we can see a trombocyte between 2 red blood cells.
- The object near two o'clock is a neutrophile.
- The object between four and five o'clock is a monocyte.
- Between the centre and ten o'clock, we can see a lymphocyte.
- At the edge, near eight o'clock, we can see part of a lymphocyte.

Systematic examination

The search for eggs and larvae of helminths and of Ciliates is performed with the 10x objective. **The entire preparation** is examined, leaving no parts missed out. To accomplish this, one should work systematically. Always start at a corner of the cover slip, e.g. the upper-left corner (in reality this will be the lower-right corner) and proceed by looking at the next microscopic field, with a small overlap. This means that each time when a field has been examined, an object in this field, e.g. a crystal, at three o'clock is chosen, and is brought towards nine o'clock. This second field is examined and so on. In this way, one should go in a straight line from the upper-left corner towards the upper-right corner of the cover slip. Once we arrive there, we choose an object at six o'clock and move it towards twelve o'clock. This results in arriving at the row below the one that has just been examined (again with a small overlap). This time we work from right to left. This way the complete preparation should be examined within the edges of the cover slip, untill arriving at the lower-right corner.





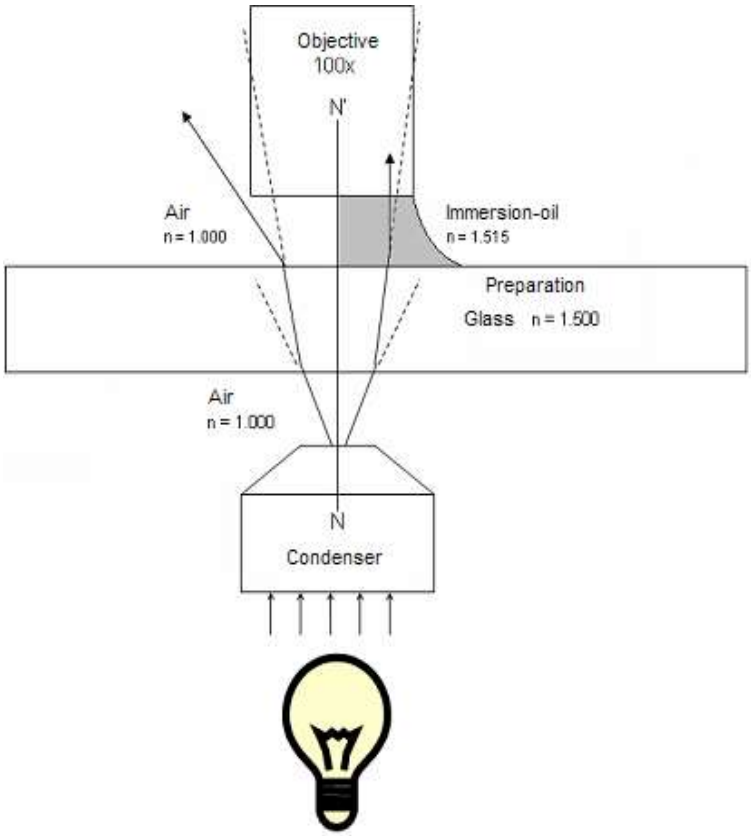
For searching some of the protozoans the 40x objective is used. In the same way as described above, a few overlapping rows (3 or 4) should be examined.
A partial systematic examination is used for permanently stained smears as well. Also here, the examined part should consist of overlapping rows.

Basic adjustments of the microscope

Objective	Condenser	Diaphragm	Immersion-oil	Mirror	Optical limit and purpose
10x	lowered	open/closed	no	flat	30 - 500 µm • Search for eggs and larvae • Search for Ciliates • (Entomology) • …
40x	halfway	open/closed	no	flat	5 - 30 µm • Details of eggs, larvae and ciliates • Search for certain protozoans by means of direct wet smear • (Counting of WBC and RBC) • …
100x	at the top	open	yes	concave	0,25 - 5 µm • Permanently stained smears • …

Immersion-objectives

Rays of light that pass the optical system of the microscope pass through different kinds of materials (glass, air, ...). Each one of these materials has a certain refracting capacity or refractional index “n”. When light passes through these materials (with different refracting indices), the rays of light will be bent and sometimes lost (when they are bent so strongly they leave the optical system of the microscope). The level and way of bending depend on the refracting capacity of the environment and on the order of the transition.
When light passes from an environment with low refractional index to an environment with higher refractional index, it will be bent to the normal NN’ of the optical system (right half of the scheme). When light passes from an environment with high refractional index to an environment with lower refractional index, it will be bent, away from the normal NN’ of the optical system (left half of the scheme).



High-magnification objectives (50x, 100x) need large amounts of light. To avoid loss of light by refraction, immersion-oil with a sufficiently high refractional index is placed between the preparation and the objective.

Some examples of different refractional indices:

Air:	n = 1,000
Water:	n = 1,330
Normal glass:	n = 1,500
Immersion-oil:	n = 1,515
Canada balm:	n = ± 1,5

Calibration of the microscope

Principals :

For the correct identification of a series of parasites, the exact dimensions are of utmost importance, especially when we are talking about cysts of protozoans and eggs of helminths. For the approximate values of a certain objects dimensions, one can start by comparing it to other elements with known dimensions (e.g. eggs of *Schistosoma mansoni*, eggs of *Trichuris trichiura*, or with red blood cells etc). One can also make an estimation of any object’s dimensions, by comparing it to the diameter of the microscopic field. To know this diameter exactly, the field-of-view-factor, mentioned on the eyepieces, is to be divided by the magnification of the used objective and by the tube-factor of the microscope. The result is expressed in millimeters. The field-of-view indicates the diameter (in mm) of the circular diaphragm, located halfway the eyepiece to limit the microscopic field. This field-of-view-factor can be found on the eyepiece, in most cases together with the magnification-factor of the eyepiece (e.g. 10x / **18**L). The tube-factor is a characteristic of the used microscope and can be found in its manual (in most cases it equals 1).

Example:
One uses eyepieces, indicating: 10x / 22.
When using a 10x objective on a microscope with tube-factor 1, this results in a diameter of the microscopic field of (22 : 10) : 1 = 2,2 mm or 2200 µm.

However, to know the exact dimensions of an object, one needs an eyepiece with a scale built inside (micrometer-eyepiece). This micrometer-eyepiece is basically a normal eyepiece, in which a lense is installed. This lense has a scale etched on it (usually divided in 100 equal parts). Since this scale is located in the eyepiece itself, it will represent different values (expressed in µm), depending on the objective being used.

This implicates however that for every different objective (and for every microscope) this micrometer-eyepiece will have to be calibrated. This means attaching a specific value (in µm) to one single division of the scale, and doing this for each objective that is used (and for each microscope). To determine this value, one needs a reference, which dimensions are known exactly. This reference usually consists of a slide on which a scale is etched (usually 1 to 2 mm), divided in 100 or 200 equal parts (each division being exactly 10 µm). Considering the high cost of these reference-slides and the limited use of them, one can replace them by a counting-chamber (without losing too much precision). Counting-chambers are for instance used in haematology to count different cell types and/or objects and can therefore be found in any routine-lab. For calibration of the 100x objective, counting chambers can not always be used as placing oil on the counting grid may render the lines of the counting grid invisible in some types of counting chambers.

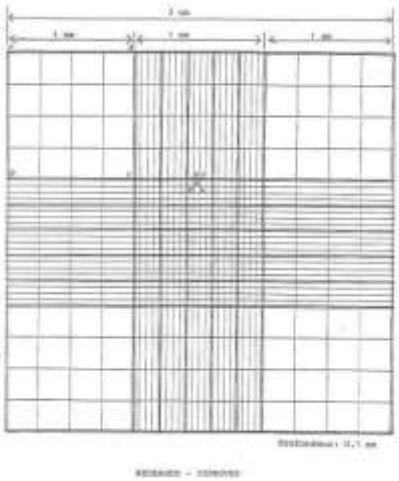
Materials and reagents :

Microscope, micrometer-eyepiece, (reference-slide or) counting-chamber, immersion-oil

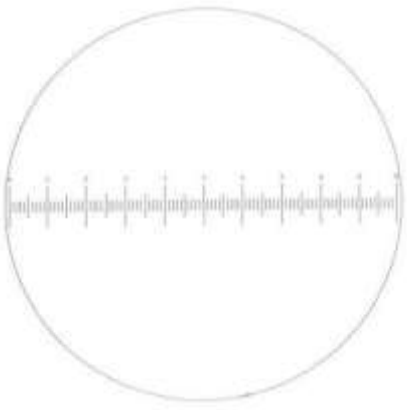
Calibration of an objective :

1. Place a counting-chamber (e.g. Neubauer Double Improved) on the mechanical stage of the microscope and manoeuvre the etched part of the counting-chamber to the centre of the microscopic field.
2. Remove the eyepiece, **of which the dioptrics are not adjustable,** and replace it by the micrometer-eyepiece.
3. Focus the microscopic image.
4. Turn the micrometer-eyepiece, placing the divisions of the scale parallel to the lines of the counting-chamber.
5. Move the counting-chamber (by moving the specimen holder) untill the 0-line of the micrometer-eyepiece corresponds exactly with a line of the counting-chamber. At high magnifications, the thickness of these lines will be such, that one should choose to make either the left or the right sides of these lines correspond.
6. Search, without moving the counting-chamber, a point at the opposite side of the microscopic field, where a division of the micrometer-eyepiece corresponds exactly with a line of the counting-chamber (as far away of the 0-line as possible).
7. Count the number of divisions of the micrometer-eyepiece between these two points of corresponding lines (in the scheme below 94 divisions of the micrometer-eyepiece can be counted).
8. Count the number of cells of the counting-chamber between the two points of corresponding lines and recalculate them to µm (in the scheme below we can see 6 cells of 250 µm + 5 cells of 200 µm, which results in a total of 2500 µm).
9. Then calculate the length of one division of the micrometer-eyepiece as follows :
   94 divisions of the micrometer-eyepiece = 2500 µm.
   1 division of the micrometer-eyepiece = 2500 / 94 = 26,6 µm.
10. Repeat all this for each objective that is to be calibrated.
11. In principle the calibration should be done only once for each microscope and for each objective that is being used. **Attention**: if the dioptrics of the micrometer-eyepiece are adjustable as well, the calibration will only be valid for de dioptrics used when calibrating!!! (The distance between eyepieces and tube influences the magnification)

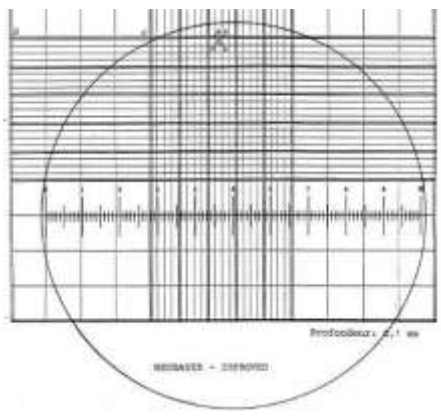
Neubauer counting-chamber (Double Improved)

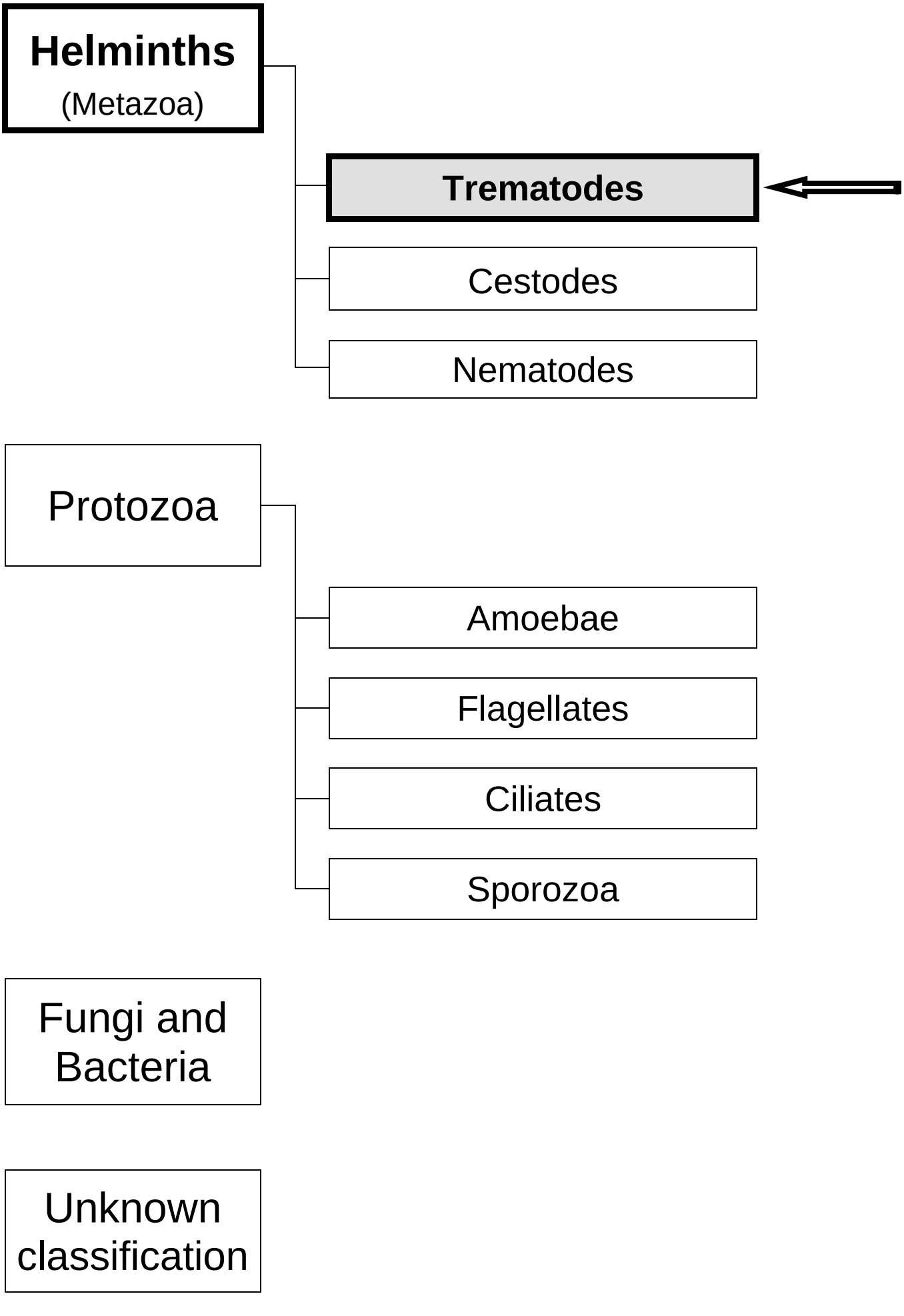


Micrometer-eyepiece

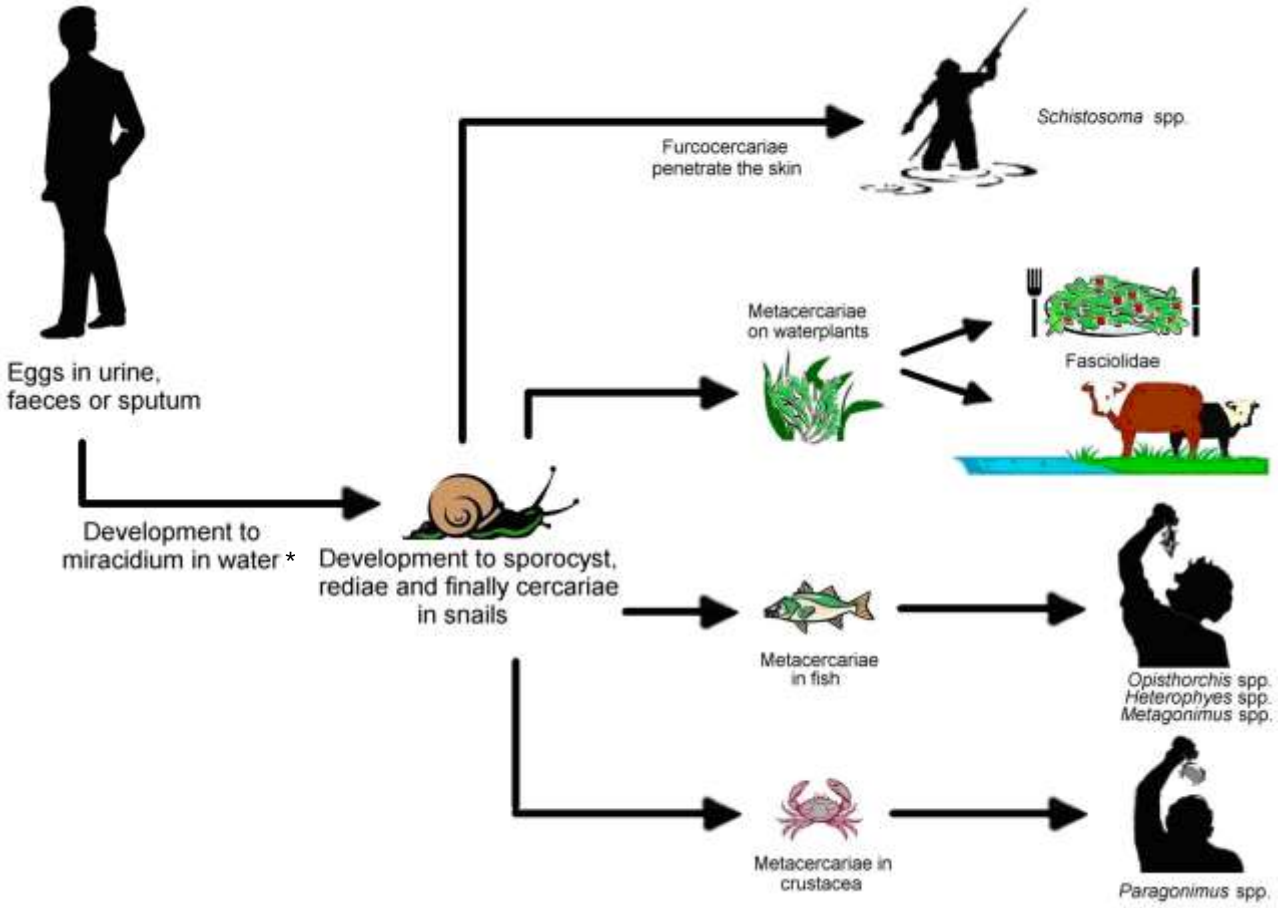


Calibration of the micrometer-eyepiece




Helminths
(Metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Example of a life-cycle: *Trematodes*



***** Except for *Opisthorchis* spp., *Heterophyes* spp. and *Metagonimus* spp., for which the eggs are to be taken up by the snail before the egg hatches and the miracidium comes out.

Schistosoma mansoni

Family : Schistosomatidae

Class : Trematodes

Geographic distribution :

- Tropical America
- Tropical Africa
- Middle East

Common name :

Intestinal bilharzia

Disease :

- NL : darm- of rectumbilharziose
- FR : bilharziose intestinale ou rectale
- EN : intestinal or rectal schistosomiasis
- ES : bilarciasis o esquistomiasis intestinal

Final host :

- Man
- Rodents (especially in Tropical America)
- Monkeys (especially in Africa)

Intermediate host :

- Freshwater snails (*Biomphalaria* spp.)

Transmission :

Transcutaneous : Furcocercariae from contaminated water will penetrate the skin (or exceptionally the mucosa).

Localisation of the adult worm :

Lower mesenterial veins.

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
  - Concentration by sedimentation
  - Kato-Katz: used only for epidemiological purposes
- Searching the eggs by rectal biopsy: the biopsy is examined without staining or fixation
- Serology (mainly based on CCA (Circulating Cathodic Antigens) and CAA (Circulating Anodic Antigens))
  - searching antibodies in serum
  - detection of immune-complexes in blood
- (Antigen-detection in **urine**: based on CCA, in Lateral Flow Through Strip or in ELISA)

Morphology of the eggs :

Dimensions : 110-175 µm x 45-70 µm

Aspect : aspect of asymmetric bottle or oval

Shell : smooth

Contents : embryo (miracidium)

Colour : rather dark

Characteristics :
big lateral spine; acid-fast egg
(red when coloured with Ziehl-Neelsen)

Associated biological signs :

- Hypereosinophilia at invasion-phase (up to 30%), which normalizes later on
- Hyperleucocytosis at prepatent phase
- Liver-tests off chart and signs of portal hypertension

Possible confusion with :

- Other *Schistosoma* spp.
- Fasciolidae
- Spores of *Psorospermium haeckelii* (sporozoa which infest crayfish in eastern Europe)

Remarks :

- Sometimes the typical lateral spine will be invisible (pointing upward or downward). In these cases the egg can be turned/rolled by gently tapping the cover slip with a pen or a wooden spatula.
- As the eggs of *S. mansoni* do not detach easily from the inner intestinal wall, repeating the examination of faeces or rectal biopsy can prove useful.
- The eggs of *S. mansoni* sometimes are found in urine-samples.
- The adult's lifespan is estimated at 2 to 18 years. Eggs can be found in faeces approximately 25 to 60 days after initial infestation. Eggs produced per female: 100 to 300 eggs per day.
- Serological tests are group-specific. Cross-reaction with other trematodes is possible. The combination of two different techniques for the detection of antibodies (ELISA + IHA) yields a sensitivity of about 90%. The antibodies can remain in circulation for years after succesful treatment.

Schistosoma japonicum

Family : Schistosomatidae

Class : Trematodes

Geographic distribution :

Far East
(China, Filippines,…)

Common name :

Intestinal bilharzia

Disease :

- NL : darmbilharziose
- FR : bilharziose intestinale
- EN : intestinal schistosomiasis
  Katayama disease
- ES : enfermedad de Katayama

Final host :

- Man
- Lots of other animals

Intermediate host :

- Freshwater snails (*Oncomelania* spp.)

Transmission :

Transcutaneous : Furcocercariae from contaminated water will penetrate the skin (or exceptionally the mucosa).

Localisation of the adult worm :

Upper mesenterial veins.

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
- Searching for eggs, using rectal biopsy: the biopsy is examined without staining or fixation
- Serology (mainly based on CCA (Circulating Cathodic Antigens) and CAA (Circulating Anodic Antigens))
  - searching antibodies in serum
  - detection of immune-complexes in blood
- (Antigen-detection in **urine**: based on CCA, in Lateral Flow Through Strip or in ELISA)

Morphology of the eggs :

Dimensions : 68-100 µm x 45-80 µm

Aspect : oval to round

Shell : smooth

Contents : embryo (miracidium)

Colour : rather light/clear

Characteristics :
small lateral spine, more or less hidden in an indentation of the egg-shell; acid-fast egg
(red when coloured with Ziehl-Neelsen)

Associated biological signs :

- Hypereosinophilia at invasion-phase, which normalises later on
- Hyperleucocytosis at prepatent phase
- Liver-tests off chart and signs of portal hypertension

Possible confusion with :

- *Schistosoma mekongi*
- *Ascaris* spp.

Remarks :

- The name Katayama Fever, originally exclusively used for infestations with *Schistosoma japonicum*, is nowadays used to indicate de acute phase of schistosomiasis in general.
- The eggs of *S. japonicum* and *S.mekongi* are morphologically identical (only small differences in dimensions). Differentiation is only possible using geographic distribution.
- The Kato-Katz-technique is not usefull as these eggs have no typical shape and the miracidium clears up quite quickly.
- The eggs of *S. japonicum* sometimes are found in urine.
- The adult's lifespan is estimated at more than 25 years. Eggs can be found in faeces approximately 30 days after initial infestation. Eggs produced per female: 1.500 to 3.500 eggs per day.

Schistosoma mekongi

Family : Schistosomatidae

Class : Trematodes

Geographic distribution :

Along the river Mekong (Laos, Cambodja, Thailand) and in Malaisia.

Common name :

Intestinal bilharzia

Disease :

- NL : darmbilharziose
- FR : bilharziose intestinale
- EN : intestinal schistosomiasis
  Katayama disease
- ES : enfermedad de Katayama

Final host :

- Man
- Lots of other animals

Intermediate host :

- Freshwater snails (*Tricula aperta*)

Transmission :

Transcutaneous : Furcocercariae from contaminated water will penetrate the skin (or exceptionally the mucosa).

Localisation of the adult worm :

Upper mesenterial veins.

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
- Searching for eggs, using rectal biopsy: the biopsy is examined without staining or fixation
- Serology (mainly based on CCA (Circulating Cathodic Antigens) and CAA (Circulating Anodic Antigens))
  - searching antibodies in serum
  - detection of immune-complexes in blood
- (Antigen-detection in **urine**: based on CCA, in Lateral Flow Through Strip or in ELISA)

Morphology of the eggs :

Dimensions : 51-73 µm x 39-66 µm

Aspect : oval to round

Shell : smooth

Contents : embryo (miracidium)

Colour : rather light/clear

Characteristics :
small lateral spine, more or less hidden in an indentation of the egg-shell; acid-fast egg
(red when coloured with Ziehl-Neelsen)

Associated biological signs :

- Hypereosinophilia at invasion-phase, which normalises later on
- Hyperleucocytosis at prepatent phase
- Liver-tests off chart and signs of portal hypertension

Possible confusion with :

- *Schistosoma japonicum*
- *Ascaris* spp.

Remarks :

- The eggs of *S .japonicum* and *S. mekongi* are morphologically identical (only small differences in dimensions). Differentiation is only possible using geographic distribution.
- The Kato-Katz-technique is not usefull as these eggs have no typical shape and the miracidium clears up quite quickly.
- The eggs of *S. mekongi* sometimes are found in urine.
- The adult's lifespan is estimated at more than 25 years. Eggs can be found in faeces approximately 30 to 60 days after initial infestation. Eggs produced per female: 1.500 to 3.500 eggs per day.

Schistosoma intercalatum

Family : Schistosomatidae

Class : Trematodes

Geographic distribution :

Central Africa.
Infestations with this parasite are usually focussed in a few villages.

Common name :

Rectal bilharzia

Disease :

- NL : intestinale / rectale bilharziose
- FR : bilharziose intestinale / rectale
- EN : intestinal / rectal schistosomiasis
- ES : bilharziasis intestinal / rectal

Final host :

- Man
- Rodents?

Intermediate host :

- Freshwater snails (*Bulinus* spp.)

Transmission :

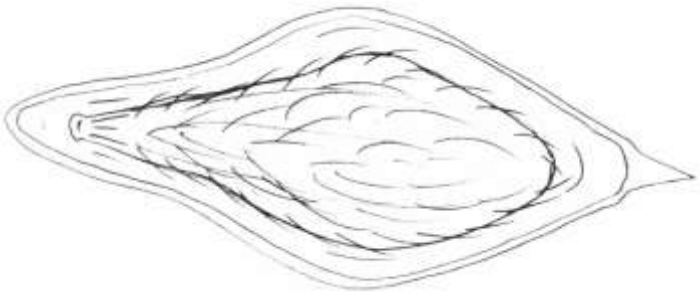
Transcutaneous : Furcocercariae from contaminated water will penetrate the skin (or exceptionally the mucosa).

Localisation of the adult worm :

Rectal veins.

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
  - Kato-Katz: used only for epidemiological purposes
- Searching for eggs, using rectal biopsy: the biopsy is examined without staining or fixation
- Serology (mainly based on CCA (Circulating Cathodic Antigens) and CAA (Circulating Anodic Antigens))
  - searching antibodies in serum
  - detection of immune-complexes in blood
- (Antigen-detection in **urine**: based on CCA, in Lateral Flow Through Strip or in ELISA)



Morphology of the eggs :

Dimensions : 140-240 µm x 50-85 µm

Aspect : usually symmetrical, stretched oval- to diamond-shaped

Shell : smooth

Contents : embryo (miracidium)

Colour : rather dark

Characteristics :
big terminal spine; acid-fast egg
(red when coloured with Ziehl-Neelsen)

Associated biological signs :

- Hypereosinophilia at invasion-phase, which normalises later on
- Hyperleucocytosis at prepatent phase
- liver-tests off chart and signs of portal hypertension

Possible confusion with :

- Other *Schistosoma* spp.
- Fasciolidae
- Spores of *Psorospermium haeckelii* (sporozoa which infest crayfish in eastern Europe)

Remarks :

- Occasionally eggs of *S. intercalatum* are found in urine samples (differentiation with *S. haematobium* using Ziehl-Neelsen's staining).
- Natural hybrids exist between *S. haematobium* and *S. intercalatum*.
- The eggs of *S. intercalatum* sometimes have difficulties detaching from the inner intestinal wall. In these cases repeating the examination of faeces or rectal biopsy can prove useful.
- Eggs can be found in faeces approximately 50 to 60 days after initial infestation.
- Eggs produced per female: 150 to 400 eggs per day.

Schistosoma haematobium

Family : Schistosomatidae

Class : Trematodes

Geographic distribution :

- Africa
- Middle East
- India
- Portugal (very rare)

Common name :

Bilharzia of the bladder

Disease :

- NL : blaasbilharziose of urinaire bilharziose
- FR : bilharziose vésicale
- EN : urinary or vesical schistosomiasis
- ES : bilarciasis urinaria

Final host :

- Man
- (Monkeys)
- (Pigs)

Intermediate host :

- Freshwater snails (*Bulinus* spp.)

Transmission :

Transcutaneous : Furcocercariae from contaminated water will penetrate the skin (or exceptionally the mucosa).

Localisation of the adult worm :

Veins of the bladder.

Diagnostic possibilities :

- Searching for eggs in urine, using: - sedimentation
  - filtration
- Serology (mainly based on CCA (Circulating Cathodic Antigens) and CAA (Circulating Anodic Antigens))
  - searching antibodies in serum
  - detection of immune-complexes in blood
- (Antigene-detection in **urine**: based on CCA, in Lateral Flow Through Strip or in ELISA)

Morphology of the eggs :

Dimensions : 112-170 µm x 40-70 µm

Aspect : symmetrically oval

Shell : smooth

Contents : embryo (miracidium)

Colour : yellow-grey

Characteristics :
small terminal spine
egg is NOT acid-fast !!!
(blue when coloured with Ziehl-Neelsen)

Associated biological signs :

- Hypereosinophilia at invasion-phase (up to 30%), which normalizes later on
- Hematuria and proteinuria
- Hyperleucocytosis at prepatent phase
- Kidney-tests off chart

Possible confusion with :

- *Schistosoma* spp. (especially *S. intercalatum*)
- Epithelial cells
- Spores of *Psorospermium haeckelii* (found in faeces !) (sporozoa which infest crayfish in eastern Europe)

Remarks :

- For specimen-collection, some points should be taken into consideration:
  - Collect the urine preferably between 10h00 and 14h00
  - Before urinating the patient should move/jump around a little bit
  - Before giving a urine-sample, the patient's bladder should be well-filled
  - As the eggs are particularly present in the last millilitres, maximum miction is advisable
- In the event of a negative result, the examination can be repeated a few days later.
- After some time the eggs of *S. haematobium* hatch and the extremely motile miracidia can be found in the urine.
- Occasionally eggs of *S. haematobium* are found in faecal samples (differentiation with *S. intercalatum* using Ziehl-Neelsen's staining).
- Natural hybrids exist between *S. haematobium* and *S. intercalatum*.
- Serological tests are group-specific. Cross-reaction with other trematodes is possible. The combination of two different techniques for the detection of antibodies (ELISA + IHA) yiels a sensitivity of about 90%. The antibodies can remain in circulation for years after succesful treatment.
- The adult's lifespan is estimated at 3 to 7 years. Eggs can be found in urine approximately 54 to 84 days after initial infestation. Eggs produced per female: 20 to 300 eggs per day.

Dicrocoelium dendriticum

Family : Dicrocoeliidae

Class : Trematodes

Geographic distribution :

Cosmopolitic

Common name :

Lancet liver fluke
(Small liver fluke)

Disease :

- NL : leverdistomatose, dicrocoeliasis
- FR : distomatose du foie, dicrocoeliase
- EN : lancet liverfluke infection, dicrocoeliasis
- ES : distomatosis hepatica, dicrocoeliasis

Final host :

Many ruminants :
- Sheep !!!
- Cow
- Goat
- …

But also:
- Pigs
- Man
- ...

Intermediate host :

1st: Terrestrial snails (*Helicella* spp., *Limicolaria* spp., *Achatina* spp.,…)
2nd: Ants (*Formica* spp., *Proformica* spp., *Lasius* spp., *Catagliphis* spp.,…)

Transmission :

By eating ants, infested with metacercariae.

Localisation of the adult worm :

Biliary channels

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
- Searching for eggs in duodenal aspirate

Morphology of the eggs :

Dimensions : 38-45 µm x 22-30 µm

Aspect : asymmetrically oval, one side slightly flattened

Shell : smooth

Contents : embryo (miracidium)

Colour : yellow to brown

Characteristics : operculum is present, but sometimes very difficult to observe

Associated biological signs :

- Hypereosinophilia
- Liver-tests off chart

Possible confusion with :

- Opisthorchidae
- *Heterophyes* spp.
- *Metagonimus* spp.

Remarks :

- Presence of eggs of *Dicrocoelium* spp. in faeces does not always indicate an infestation however. The possibility exists that these are passing eggs (eating of infected liver → spurious infection or "pseudo-dicrocoeliasis").
- Eggs can be found in faeces approximately 8 to 14 weeks after initial infestation.
- The eggs of *D. dendriticum* are resistant to desiccation and survive freezing temperatures.
- *D. dendriticum* (syn: *D. lanceolatum*, *D. lanceatum*) and *D. hospes* are morphologically identical. *D. hospes* occurs more often in cattle in Africa.
- The cercariae of *Dicrocoelium* clearly alter the behaviour of the ants they use as intermediate hosts: infested ants crawl to the tops of blades of grass when temperatures drop below 20°C (i.e. from dusk untill early morning, the two periods during which herbivores are grazing). Here they cling as if paralysed, using their mandibles. This strange behaviour makes the ants much more likely to be eaten by a grazing animal.

Fasciola hepatica

Family :	**Class :**
Fasciolidae	Trematodes

Geographic distribution :	**Common name :**	**Disease :**
Worldwide	Liverfluke	• NL : leverdistomatose • FR : distomatose du foie • EN : fasciolasis, liver fluke infection • ES : distomatosis hepatica

Final host :	**Intermediate host :**	**Transmission :**
Many vegetarians : • Sheep • Goat • Cow • Man • Rodents • …	• Amphibic freshwater snails (*Lymnea* spp.)	By eating waterplants, contaminated with metacercariae. **Localisation of the adult worm :** Biliary channels

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  Usually the number of eggs in the faeces is rather low so a direct examination will not be sufficient.
  - **Concentration by sedimentation**
- Searching for eggs in duodenal aspirate
- Serology: searching for antibodies (against excretion and secretion Ag (ES Ag)) in serum
- (Antigene-detection in faeces (ES Ag))

Morphology of the eggs :

Dimensions : 120-150 µm x 63-90 µm
Aspect : symmetrically oval
Shell : smooth and thin wall
Contents : no embryo
Colour : yellow, brown-yellow or brown-grey
Characteristics : operculum is present, but sometimes very difficult to observe

Associated biological signs :

- Hyperleucocytosis in invasion-phase
- Hypereosinophilia (up to 80% at invasion-phase)
- Liver-tests off chart

Possible confusion with :

- *Fasciola gigantica, Fasciolopsis buski*
- *Echinostoma ilocanum*
- *Gastrodiscoides hominis*
- *Schistosoma* spp.
- eggs of *Acarina* spp. (mites)

Remarks :

- The distinction between eggs of *Fasciola hepatica, Fasciola gigantica* and *Fasciolopsis buski* is microscopically almost impossible to make. There are natural hybrids as well between *F. hepatica* and *F. gigantica*, making it impossible to differentiate these two eggs. When one of these eggs is found in a faecal sample, a more correct answer would be *"*Eggs of Fasciolidae found*"*.
- Also the eggs of *Echinostoma ilocanum* and *Gastrodiscoides hominis* resemble those of the Fasciolidae and are very difficult to differentiate.
- Presence of eggs of *Fasciola hepatica* in faeces does not always indicate an infestation however. The possibility exists that these are passing eggs (eating of infected liver → spurious infection or "pseudo-fasciolasis").
- Soon after initial infestation (7 to 11 weeks) the larvae penetrating the parenchym of the liver will cause a pathology. At this point no eggs can be found in the faeces, but serology can be helpful: 2 to 4 weeks after infestation serology has a sensitivity of about 95% (there will be cross-reactivity with other trematodes). Approximately 1 year after the invasion-phase serology can become negative again (while the adult worms are still in the biliary channels).
- When the operculum is not visible, one can firmly press on the cover slip with a wooden spatula, trying to open the operculum in this manner.
- The adult's lifespan is estimated at more than 25 years. Eggs can be found in faeces approximately 11 to 17 weeks after infestation. The number of eggs produced daily per adult is rather high (± 25.000 eggs per adult). Probably, only a very limited number of these eggs can eventually be found in the patient's faeces.

Fasciola gigantica

Family : Fasciolidae

Class : Trematodes

Geographic distribution :

- Central- and South-Africa
- (West-Pacific)
- (Haïti)
- (Asia)

Common name :

Big liverfluke

Disease :

- NL : leverdistomatose
- FR : distomatose du foie
- EN : fasciolasis, giant liver fluke infection
- ES : distomatosis gigantica

Final host :

Different kinds of plant-eaters :

- Sheep
- Goats
- Cows
- Man
- Rodents
- …

Intermediate host :

- Aquatic freshwater snails (*Lymnea* spp.)

Transmission :

By eating waterplants, contaminated with metacercariae.

Localisation of the adult worm :

Biliary channels

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
    Usually the number of eggs in the faeces is rather low so a direct examination will not be sufficient.
  - Concentration by sedimentation
- Searching for eggs in duodenal aspirate
- **Serology: searching for antibodies (against excretion and secretion Ag (ES Ag)) in serum**
- (Antigene-detection in faeces (ES Ag))

Morphology of the eggs :

Dimensions : 135-190 µm x 68-94 µm
Aspect : symmetrically oval
Shell : smooth and thin wall
Contents : no embryo
Colour : yellow, brown-yellow or brown-grey
Characteristics : operculum is present, but sometimes very difficult to see

Associated biological signs :

- Hyperleucocytosis in invasion-phase
- Hypereosinophilia (up to 80% in invasion-phase)
- Liver-tests off chart

Possible confusion with :

- *Fasciola hepatica, Fasciolopsis buski*
- *Echinostoma ilocanum*
- *Gastrodiscoides hominis*
- *Schistosoma* spp.
- Eggs of *Acarina* spp. (mites)

Remarks :

- The distinction between eggs of *Fasciola hepatica, Fasciola gigantica* and *Fasciolopsis buski* is microscopically almost impossible to make. There are natural hybrids as well between *F. hepatica* and *F. gigantica*, making it impossible to differentiate these two eggs. When one of these eggs is found in a faecal sample, a more correct answer would be *"*Eggs of Fasciolidae found*"*.
- Also the eggs of *Echinostoma ilocanum* and *Gastrodiscoides hominis* resemble those of the Fasciolidae and are very difficult to differentiate.Presence of eggs of Fasciolidae in faeces does not always indicate an infestation however. The possibility exists that these are passing eggs (eating of infected liver → spurious infection or "pseudo-fasciolasis").
- Soon after initial infestation (7 to 11 weeks) the larvae penetrating the parenchym of the liver will cause a pathology. At this moment no eggs can be found in the faeces, but serology can be helpful: 2 to 4 weeks after infestation serology has a sensitivity of about 95% (there will be cross-reactivity with other trematodes). Approximately 1 year after the invasion-phase serology can become negative again (while the adult worms are still in the biliary channels).
- When the operculum is not visible, one can firmly press on the cover slip with a wooden spatula, trying to open the operculum in this manner.
- In human infestations, the adults of *Fasciola gigantica* are usually sterile. As no eggs can be found in this case, serology can prove useful. If the adults aren't sterile, eggs can be found in the patient's faeces approximately 12 to 15 weeks after infestation. Probably, only a limited number of eggs produced can eventually be found in the patient's faeces. The adult's lifespan is estimated at more than 11 years.

Fasciolopsis buski

Family : Fasciolidae

Class : Trematodes

Geographic distribution :

- China
- South-East Asia
- Far East
- Central Europe
- Indian continent

Common name :

Intestinal fluke

Disease :

- NL : darmdistomatose
- FR : distomatose intestinale
- EN : fasciolopsiasis
  Busk's fluke infection
- ES : distomatosis intestinal

Final host :

- Man
- Pig
- Dog (rare)
- Monkey
- Rabbit

Intermediate host :

- Freshwater snails (*Hippeutis* spp., *Segmentina* spp.)

Transmission :

By eating waterplants, contaminated with metacercariae.

Localisation of the adult worm :

Intestines (sucked to the wall of the ileum)

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
  - Concentration by sedimentation

  As there is no pathology with low wormloads and as egg-production is rather high, a concentration-technique should not be considered.

Morphology of the eggs :

Dimensions : 130-159 µm x 78-98 µm

Aspect : symmetrically oval

Shell : smooth and thin wall

Contents : no embryo

Colour : yellow, brown-yellow or brown-grey

Characteristics :
operculum is present, but sometimes very hard to see

Associated biological signs :

- Hypereosinophilia

Possible confusion with :

- *Fasciola hepatica, Fasciola gigantica*
- *Echinostoma ilocanum*
- *Gastrodiscoides hominis*
- *Schistosoma* spp.
- Eggs of *Acarina* spp. (mites)

Remarks :

- The distinction between eggs of *Fasciola hepatica, Fasciola gigantica* and *Fasciolopsis buski* is microscopically almost impossible to make. There are natural hybrids as well between *F. hepatica* and *F. gigantica*, making it impossible to differentiate these two eggs. When one of these eggs is found in a faecal sample, a more correct answer would be *"*Eggs of Fasciolidae found*"*.
- Also the eggs of *Echinostoma ilocanum* and *Gastrodiscoides hominis* resemble those of the Fasciolidae and are very difficult to differentiate.
- Rare cases of ectopic localisations (in myocard or brain) have been reported in literature.
- When the operculum is not visible, one can firmly press on the cover slip with a wooden spatula, trying to open the operculum in this manner.
- The adult's lifespan is estimated at about 6 months.
- Eggs can be found in faeces approximately 12 to 20 weeks after infestation.
- Eggs produced per adult: ±16.000 eggs per day.

Opisthorchis sinensis

Family : Opisthorchidae

Class : Trematodes

Geographic distribution :

- Far East (Japan, Korea, Hong-Kong, Taiwan, China, Kamchatka,…)

Common name :

Chinese liverfluke

Disease :

- NL : Chinese leverdistomatose
- FR : distomatose de Chine
- EN : Chinese liverfluke infection, clonorchiasis
- ES : distomatosis hepatica chinesco

Final host :

- Man
- Many fish-eating mammals

Intermediate host :

1st: Freshwater snails (*Bithynia* spp., *Alocinma* spp., *Parafossarulus* spp.)

2nd: Freshwater fish

Transmission :

By eating fish, contaminated with living metacercariae.

Localisation of the adult worm :

Biliary channels

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
  - Concentration by sedimentation
- Searching for eggs in duodenal aspirate
- Serology: searching for antibodies in serum (ELISA)

Morphology of the eggs :

Dimensions : 26-35 µm x 12-14 µm

Aspect : shape of asymmetrical amphora or stretched oval

Shell : smooth

Contents : embryo (miracidium)

Colour : brown-yellow

Characteristics : small knob (not always visible) on the pole, opposite to the well-visible operculum

Associated biological signs :

- Hypereosinophilia (up to 20%)

Possible confusion with :

- Other Opisthorchidae
- *Heterophyes* spp.
- *Metagonimus* spp.

Remarks :

- *Opisthorchis viverrini* (South-East Asia and Malaisia), *O. felineus* (*tenuicollis*) (South-East and Central Europe), etc. are liverflukes that have a biology and pathology similar to *O. sinensis*. Also their eggs (which can be found in faecal material) are practically indistinguishable.
- Serology is not very specific.
- The eggs of *Heterophyes heterophyes,* of *Metagonimus yokogawai* and of 13 other species of Heterophyidae that have been described (and which can also be found in faecal material) have a strong resemlance to these of the Opisthorchidae. The adult worms of *H. heterophyes* and of *M. yokogawai* live in the intestinal lumen however, causing only a very mild pathology (sometimes even asymptomatic). Their lifespan as adult worms is limited to about one year (while this is sometimes over 20 years for the Opisthorchidae).
- Smoking and/or pickling doesn't always kill the metacercariae.
- The adult's lifespan is estimated at more than 25 years.
- Eggs can be found in faeces approximately 1 to 4 weeks after infestation.
- Eggs produced per adult: 1.000 to 4.000 eggs per day.

Paragonimus spp.

Family : Troglotrematidae

Class : Trematodes

Geographic distribution :

- Sout-East Asia
- Africa
- America

Common name :

Lungfluke

Disease :

- NL : longdistomatose
- FR : distomatose pulmonaire
- EN : pulmonary distomatosis, oriental lungfluke disease
- ES : distomatosis pulmonar, duela pulmonar

Final host :

- Man
- Lots of different animals
  - Dog
  - Cat
  - Pig
  - Apes
  - …

Intermediate host :

1st: Freshwater snails (*Thiaridae* spp., *Oncomelania* spp.,…)

2nd: Freshwater crustaceans (lobster, crab,…)

Transmission :

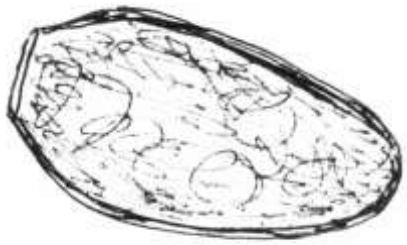
By eating crustaceans, infested with living metacercariae (raw, pickled, crab-meat as an aperitif,…)

Localisation of the adult worm :

Lungs (ectopic localisations are possible)

Diagnostic possibilities :

- Searching for eggs in sputum, using: concentration by sedimentation (after liquification)
  In case of high wormloads the sputum will often have brown to red dots in it, corresponding with important egg-masses.
- Searching for specific antibodies (against *Paragonimus westermani*) in blood



Morphology of the eggs :

Dimensions : 68-118 µm x 39-67 µm

Aspect : (sometimes irregularly) oval

Shell : thin shell with irregularities

Contents : no embryo

Colour : dork brown-yellow to greyish

Characteristics : operculum (sometimes hard to see)

Associated biological signs :

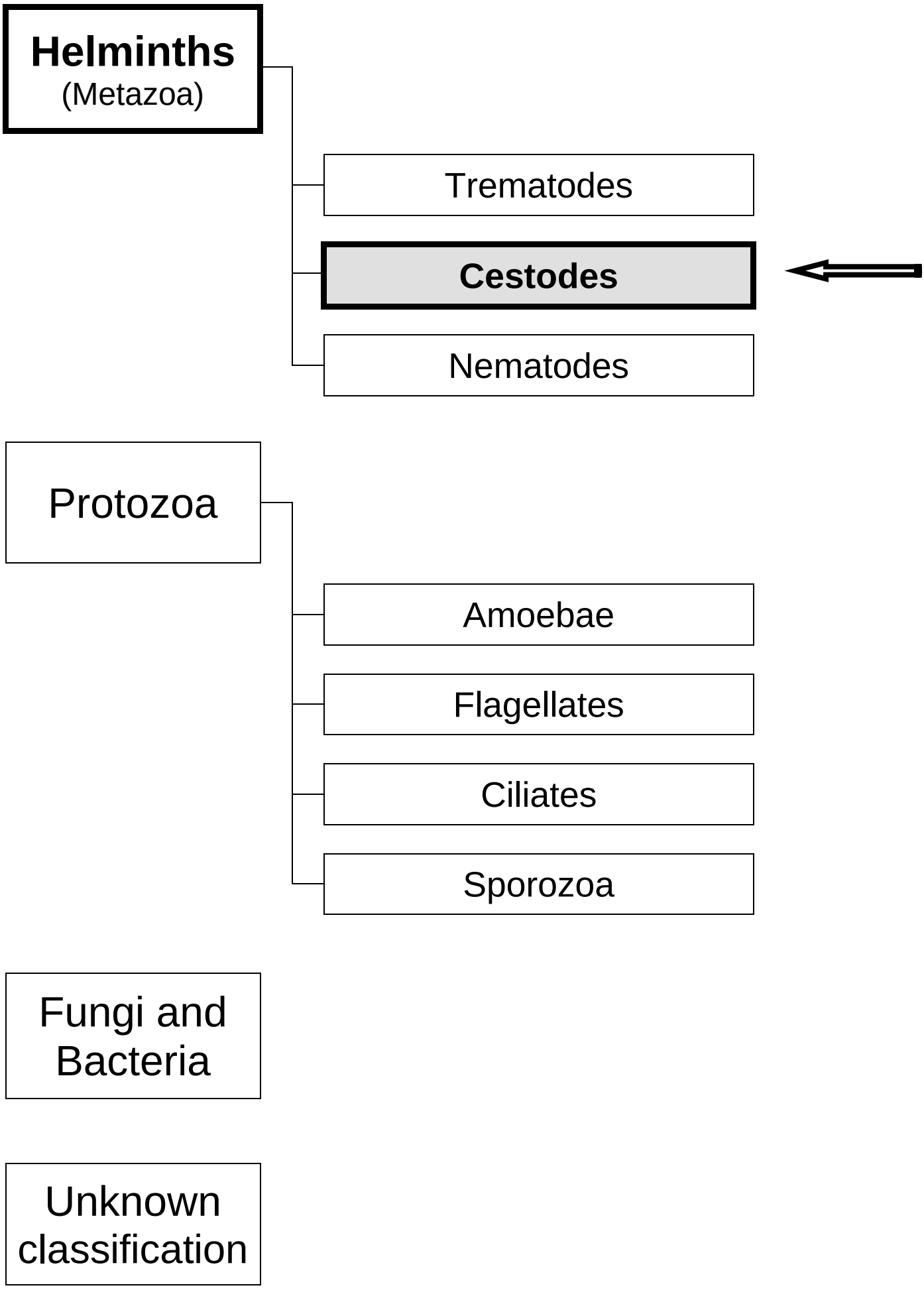
- Hypereosinophilia (in invasion-phase)
- Coughing up bloody sputum (chronic phase)

Possible confusion with :

- Eggs of *Diphyllobothrium latum* (in faeces)
- Eggs of *Ascaris* spp. (non fertile) (in faeces)
- Eggs of *Schistosoma mekongi* (in faeces)
- Eggs of *Schistosoma japonicum* (in faeces)
- Eggs of Fasciolidae (in faeces)

Remarks :

- One should never do a parasitological examination of (bloody) sputum before excluding a TB infection (risk of producing infectious aerosols).
- Eggs can be found in sputum only 2 to 3 months after infestation. The adult's lifespan is estimated at 10 to 20 years.
- Detection of eggs in sputum is not very easy, making serology particularly interesting, especially in case of low parasitaemias.
- When the operculum is not visible, one can firmly press on the cover slip with a wooden spatula, trying to open the operculum in this manner, thus making it visible and confirming the diagnosis.
- When examining sputum larvae can sometimes be found of worms, passing the lungs in their cycle of development (e.g. *Ascaris lumbricoides,* Hookworms*, Strongyloides stercoralis,*...). Examination of sputum however is not the best way to search for these helminths!
- Examination of faeces is not indicated: the swallowed eggs will be passed in the faeces, but these will be hard to identify and very few in number. However, if these eggs are found in faeces, a sputum examination is indicated for confirmation.
- There is no morphological distinction possible between the different species of *Paragonimus* (*P. westermani, P.heterotremus, P. miyazakii, P. skyrjabini* (Asia, Africa?, South-America?), *P. africanus, P. uterobilateralis* (Africa), *P. kellicotti* (North-America), *P. mexicanus* (Latin America),…). The most correct answer to give when these eggs are found, is "eggs of *Paragonimus* spp. found".
- So far, the serological tests have no standardized protocol.


Helminths
(Metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Diphyllobothrium latum

Family : Diphyllobothriidae

Class : Cestodes

Geographic distribution :

Cosmopolitic, more frequent in regions with temperate climate:
- North- and Central-Europe
- Japan, Taiwan, Filippines
- North- en South-America

Common name :

Fish tapeworm

Disease :
- NL : dibotriocefalose
- FR : diphyllobothriose humaine bothriocéphalose
- EN : broad fish tapeworm infection
- ES : botriocéfalosis

Final host :
- Man
- Many fish-eating mammals

Intermediate host :

1st: Small Copepoda (*Cyclops* spp., *Diatomus* spp.,…)

2nd: Freshwater fish (salmon, trout,…)

Transmission :

By eating fish, contaminated with living, plerocercoid larvae.

Localisation of the adult worm :

Attached to the inner wall of the small intestine

Diagnostic possibilities :
- Searching for eggs in faeces, using: - Direct examination
  As the number of eggs, produced by *D. latum* is quite high, a concentration-technique is usually not necessary.
- Identification of segments found in faeces (very rare)

Morphology of the eggs :

Dimensions : 58-76 µm x 40-51 µm
Aspect : symmetrically oval
Shell : thin
Contents : no embryo
Colour : rather dark
Characteristics : small knob (not always visible) on the opposite pole of the usually inconspicuous operculum.

Associated biological signs :
- Megaloblastic macrocytic anaemia (Vitamine B12 deficiency)
- Leucopenia, thrombopenia, non-constant hypereosinophilia
- Elevated sedimentationspeed, hypoproteinemia, hypoalbuminemia, hypergammaglobulinemia

Possible confusion with :
- *Paragonimus* spp.

Remarks :
- Freezing of fish, contaminated with plerocercoid larvae, at -10°C during at least 24h, will kill these larvae.
- Sparganosis (or plerocercoidosis) is a disease in which *Diphyllobothrium* spp., using exclusively animals as a final host, are using humans as an unusual, paratenic (=no complete development in the host) intermediate host. In this case, only pro- or plerocercoid larvae are found in human host tissues. The localisation of these larvae will determine whether the course of the disease is rather mild and/or unnoticed (e.g. larvae in intestinal wall) or serious and/or dangerous evolution (e.g. larvae in ocular tissue). In cases of sparganosis no eggs are found in the faeces!
- Mature (non-motile) segments usually dissolve inside the intestins and hence will not be found in the faeces.
- Smoking or pickling of contaminated fish will not always kill the larvae!
- About 50 different *Diphyllobothrium* species have been recorded, of which 13 can infest humans.
- *D. latum, D. cordatum* (Northern-Europe, Northern-America,...), *D. pacificum* (South-Amerika),... are morphologically indistinguishable.
- The adult's length can be up to 12 m and its lifespan is estimated at up to 30 years.
- Eggs can be found in faeces approximately 30 to 45 days after initial infestation.
- Eggs produced per adult: 35.000 to 1.000.000 eggs per day.

Taenia saginata

Family :	**Class :**
Taeniidae	Cestodes

Geographic distribution :

Cosmopolitic; rare in countries where no beef is eaten (hindu) or where systematic veterinary controls are conducted

Common name :

Unarmed tapeworm
Beef tapeworm

Disease :

- NL : teniasis
- FR : téniase
- EN : beef tapeworm infection
- ES : teniasis

Final host :

- Man

Intermediate host :

- Bovidae (cow, ox, buffalo, zebu,...)

Transmission :

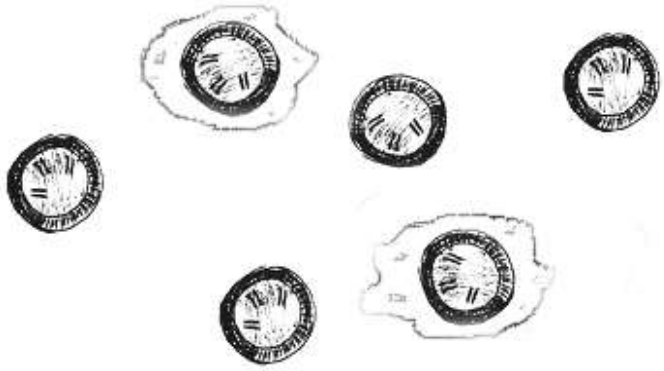
By eating beef, contaminated with living cysticerci (*Cysticercus bovis*).

Localisation of the adult worm :

Attached to the inner wall of the small intestine

Diagnostic possibilities :

- Macroscopically (finding the segments of the tapeworm)
- Microscopically by finding the eggs in the faeces: - Direct examination
  - Concentration by sedimentation
- The tape-test (for *Enterobius vermicularis*) can give an accidental diagnosis (eggs on peri-anal skin)
- (Searching copro-antigenes with dipsticks (good sensitivity, but no species-differentiation possible))
- (Serology: searching for specific antibodies)
- (Searching for specific DNA by PCR on faecal extractions (species-differentiation possible))
- **CAUTION:** When manipulating segments or adult tapeworms, always proceed with lots of caution (risk of cysticercosis in case of *T. solium*). Manipulation of segments or adult tapeworms is best done using tweezers. ALWAYS wear protective gloves when manipulating segments or adult tapeworms.



Morphology of the eggs :

Dimensions : 31-43 µm
Aspect : round or oval
Shell : thick wall with radial structure
Contents : hexacanth larva (3 pairs of hooks, all of which are not always visible)
Colour : dark-brown
Characteristics : sometimes some sort of mucus (periovular membrane) can be observed around the egg

Associated biological signs :

- At first: moderate hypereosinophilia (± 20%)

Possible confusion with :

- Air-bulbs
- *Taenia solium, Taenia asiatica*
- Different kinds of pollen-grains

Remarks :

- In french the tapeworm is sometimes called “ver solitaire” (solitary worm), because it was thought that a person could be infected with only 1 tapeworm at a time. It has been proven several times already that a patient can bear more than one tapeworm at a time however.
- The eggs of all human *Taenia* spp. are morphologically identical. When one of these is found in an examination of faeces the most correct answer should be “eggs of *Taenia* sp. found”.
- The microscopic examination (for eggs) of the faeces usually stays negative (because usually intact segments are excreted, even in between defecations).
- The difference between *T. saginata* and *T.* solium can be made by looking at mature segments or by looking at the scolex.
- *T. asiatica* is notoriously difficult to distinguish from *T. saginata*. It was formerly known as *T. saginata taiwanensis* or *T. saginata asiatica* but is now accepted as a separate species. The adult’s scolex possesses rudimentary hooklets in a wart-like formation. It uses pigs as an intermediate host. *T. asiatica* is only found in Asia (Republic of Korea, China, Taiwan, Indonesia, and Thailand).
- Cysticerci of *Taenia saginata* in meat can remain alive for 8 months to one year, but can be killed by thorougly cooking the meat or by freezing it (at least 24h at –20°C).
- The first 10 to 12 weeks after infestation no segments or eggs will be found in the faeces (however, at this moment there will be a hypereosinophilia).
- Each mature segment contains up to 100.000 eggs. One single tapeworm (counting as many as 2000 segments) can produce hundreds of millions of eggs each year, while the adult’s lifespan is estimated at more than 35 years.

Taenia solium

Family : Taeniidae

Class : Cestodes

Geographic distribution :

Cosmopolitic; rare in countries where no pork is eaten (moslims) or where systematic veterinary controls are conducted

Common name :

Armed tapeworm
Pork tapeworm

Disease :

- NL : teniasis, (neuro)cysticercose
- FR : téniase, (neuro)cysticercose
- EN : pork tapeworm infection, cysticercosis
- ES : teniasis, cisticercosis

Final host :

- Man

Intermediate host :

- Pig
- Occasionally man, with very serious pathology (cysticercosis)
- Dog, cat, ovidae and primates

Transmission :

- By eating pork, contaminated with living cysticerci (*Cysticercus cellulosae*).
- By ingestion of the eggs (faeco-oral or by anti-peristaltism of mature segments) → cysticercosis

Localisation of the adult worm :

Attached to the inner wall of the small intestine

Diagnostic possibilities :

- Macroscopically (finding the segments of the tapeworm)
- Microscopically by finding the eggs in the faeces: - Direct examination
  - Concentration by sedimentation
- (The tape-test (for *Enterobius vermicularis*) can give an accidental diagnosis (eggs on peri-anal skin))
- (Searching copro-antigenes with dipsticks (good sensitivity, but no species-differentiation))
- (Serology: searching for specific antibodies by Western Blotting of serum or CSF (only in case of cysticercosis))
- (Searching for specific DNA by PCR on faecal extractions (with species-differentiation))
- Medical Imaging (only in case of cysticercosis)
- **CAUTION:** When manipulating segments or adult tapeworms, always proceed with lots of caution (risk of cysticercosis in case of *T. solium*). Manipulation of segments or adult tapeworms is best done using tweezers. ALWAYS wear protective gloves when manipulating segments or adult tapeworms.

Morphology of the eggs :

Dimensions : 31-43 µm

Aspect : round or oval

Shell : thick wall with radial structure

Contents : hexacanth larva (3 pairs of hooks, all of which are not always visible)

Colour : dark-brown

Characteristics : sometimes some sort of mucus (periovular membrane) can be observed around the egg

Associated biological signs :

- At first: moderate hypereosinophilia (± 20%)

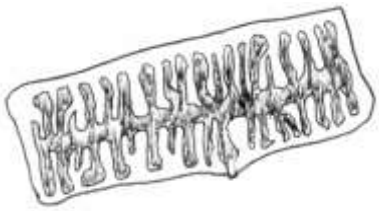
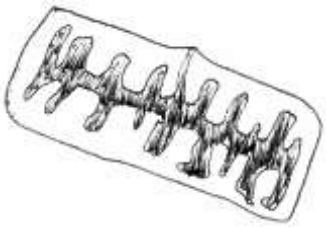
Possible confusion with :

- Air-bulbs
- *Taenia saginata, Taenia asiatica*
- Different kinds of pollen-grains

Remarks :

- In french the tapeworm is sometimes called “ver solitaire” (solitary worm), because it was thought that a person could be infected with only 1 tapeworm at a time. It has been proven several times already that a patient can bear more than one tapeworm at a time however.
- The eggs of all human *Taenia* spp. are morphologically identical. When one of these is found in a examination of faeces the most correct answer should be “eggs of *Taenia* sp. found”.
- The difference between *T. saginata* and *T.* solium can be made by looking at mature segments or by looking at the scolex.
- Cysticerci in meat can remain alive for 8 months to one year, but can be killed by thorougly cooking the meat or by freezing it (at least 24h at –20°C). The eggs of *Taenia solium* can remain viable for at least 8 months.
- The first 5 to 12 weeks after infestation no segments or eggs will be found in the faeces (however, at this moment there will be a hypereosinophilia). The microscopic examination (for eggs) of the faeces usually stays negative (because usually intact segments are excreted with the faeces).
- In cases of cysticercosis cysticerci can be found in:
  - brain-tissue (cerebral) in 40% of the cases → hypereosinophilia
  - other cases are ocular (with moderate hypereosinophilia), muscular or sub-cutaneous (most of the time asymptomatic).
- Serological tests for cysticercosis can be negative, especially in cases of older infestations. Their sensitivity is about 90%, while having a specificity of about 97%.
- Each mature segment contains about 50.000 eggs. One single tapeworm (counting as many as 1000 segments) can produce hundreds of millions of eggs each year, while the adult’s lifespan is estimated at more than 25 years.

Differential diagnosis of T. saginata / T. solium

Taenia saginata	***Taenia solium***
Scolex : Unarmed tapeworm  Diameter: 1-2 mm 4 suckers No rostrum No hooks	**Scolex :** Armed tapeworm  Diameter: 1 mm 4 suckers Rostrum with hooks
Length of the adult worm : Average of 5 to 10 m	**Length of the adult worm :** Average of 3 to 5 m
Mature segment :  Very motile at excretion. 15 to 32 fine, unilateral ramifications. Length of each segment: 12-15 mm	**Mature segment :**  Almost immobile at excretion. 7 to 16 larger, unilateral ramifications. Length of each segment: 10-12 mm
Morphology of the cysticercus : *Cysticercus bovis* (in cow) in muscular tissue. No hooks. Usually few cysticerci present. 	**Morphology of the cysticercus :** *Cysticercus cellulosae* (in pig and man) in muscular tissue or in the central nervous system. Hooks present. Usually lots of cysticerci present. 

Diagnostic possibilities :

- Macroscopically (finding the mature segments of the tapeworm):
  - Very motile segment, excretion in between defecations: *Taenia saginata*
  - Less motile segment / not excreted in between defecations:
    Differentiation is made by counting the ramifications of the central uterus-canal. To visualise these ramifications, the segment is pressed between two glass slides and looked at in front of a light-source, if necessary, after treating it with acetic acid or glycerine. When treating the segments, always treat more than one segment in regard of the possible overlap (15-16) of ramifications.

CAUTION: When manipulating segments or adult tapeworms, always proceed with lots of caution (risk of cysticercosis in case of *T. solium*). Manipulation of segments or adult tapeworms is best done using tweezers. ALWAYS wear protective gloves when manipulating segments or adult tapeworms.

Hymenolepis nana

Family : Hymenolepididae

Class : Cestodes

Geographic distribution :

Cosmopolitic, but more frequent in:
- Eastern and Southern Europe
- India
- America
- Northern Africa

Common name :

Dwarf tapeworm

Disease :
- NL : hymenolepiasis
- FR : hyménolépiase
- EN : dwarf tapeworm infection, hymenolepiasis
- ES : himenolepiasis

Final host :
- Man
- (Rodents)

Intermediate host :

H. nana is the only tapeworm infecting man, which has NO intermediate host.

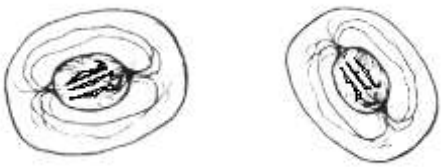
Vector: flour-worms ?

Transmission :
- Oral ingestion of the eggs.
- (Oral ingestion of flour-worms, infected with cysticercoid larvae)

Localisation of the adult worm :

Attached to the inner wall of the ileum

Diagnostic possibilities :
- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
- (Identification of the adult worm in faeces)



Morphology of the eggs :

Dimensions : 40-60 µm x 30-50 µm

Aspect : symmetrically oval or round

Shell : thin, smooth

Contents : embryophore (with 2 polar knobs and a few filaments) containing a hexacanth larva with well-visible hooks

Colour : transparent, colourless

Associated biological signs :
- Moderate hypereosinophilia

Possible confusion with :
- *Hymenolepis diminuta*
- *Hymenolepis fraterna*
- Pollen grains (barley)

Remarks :
- *Hymenolepis nana* is also known as *Vampirolepis nana*.
- The adult worm measures 1,5 to 4,0 cm, has 4 suckers and a scolex with numerous hooks.
- Mature segments already decompose inside the intestine and thus will not be found in the faeces.
- *Hymenolepis fraterna* is a similar cestode. Its final hosts are mainly rodents, but sometimes man can also be the final host. It needs an intermediate host however (fleas, cockroaches, flour-worms,...). The eggs of *H. fraterna* are morphologically identical to those of *H. nana.* When one of these eggs is found in a faeces sample the most correct answer should be "*eggs of Hymenolepis spp. found*".
- As only high levels of infestation will cause a pathology, concentration-techniques are of lesser importance.
- The possibility of auto-infestation (mainly with children) usually causes high levels of infestation, causing concentration-techniques to be less useful.
- The adult's lifespan is estimated at only a few months.
- Eggs appear in faeces about 20 days after initial infestation.

Hymenolepis diminuta

Family : Hymenolepididae

Class : Cestodes

Geographic distribution :

Cosmopolitic, but very rare (usually in regions with habitats in poor condition, where rodents have acces to food of man)

Common name :

Small tapeworm
Rat tapeworm

Disease :

- NL : hymenolepiasis
- FR : hyménolépiase
- EN : rat tapeworm infection hymenolepiasis
- ES : himenolepiasis

Final host :

- Rodents
- Man (zoonosis)

Intermediate host :

All kinds of arthropodes :

- Flea of rats
- Flour-worms
- Dung-beetles
- …

Transmission :

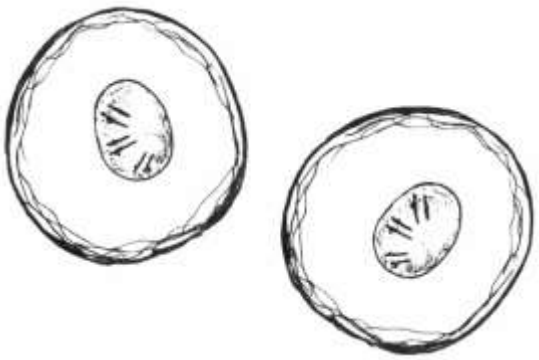
Accidental ingestion of insects, infested with cysticercoid larvae of *H. diminuta*.

Localisation of the adult worm :

Attached to the inner wall of the jejunum.

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
  - (Concentration by flottation)
- (Identification of the adult worm in faeces)



Morphology of the eggs :

Dimensions : 70-86 µm x 60-80 µm

Aspect : usually round

Shell : rather thick and smooth

Contents : embryophore without polar knobs, containing a hexacanth larva with well-visible hooks; between the embyophore and the shell a filamentous mass can be observed, pressed against the outer wall.

Colour : brown to brown-yellow

Associated biological signs :

- Moderate hypereosinophilia

Possible confusion with :

- *Hymenolepis nana*
- *Hymenolepis fraterna*
- Spores of laurel and crocus

Remarks :

- Mature segments usually dissolve inside the intestine and hence will not be found in the faeces.
- The adult worm has a length of about 20 to 60 cm, has 4 suckers and does not possess hooks.
- Untill 2005, worldwide only less than 100 cases of infestation with *H. diminuta* were reported.
- Eggs appear in faeces about 20 days after initial infestation.
- The adult's lifespan is estimated at about 5 to 7 weeks.

Echinococcus granulosus

Family :	**Class :**
Taeniidae	Cestodes

Geographic distribution :

Cosmopolitic, especially in regions with lots of ovidae and bovidae.

Common name :

Hydatic cyst

Disease :

- NL : echinococcosis, hydatidose
- FR : échinococcose, hydatidose
- EN : echinococcosis, hydatidosis, hydatid disease
- ES : hidatidosis

Final host :

- Carnivores, **except man**

Intermediate host :

- Herbivores (especially sheep)
- Cow, horses,…
- **Man**

Transmission :

By ingestion of eggs (contact with carnivores (e.g. dog) or with contaminated food).

Localisation of the adult worm :

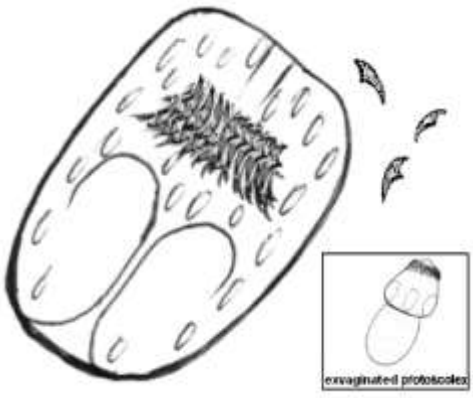
In the intestinal lumen of different kinds of carnivores (**except humans**).

Localisation of the hydatic cyst :

Usually hepatic (75%), sometimes pulmonary (10%), muscular (5%), splenic (3%), osseous (2%), renal (2%) or in other organs (3%).

Diagnostic possibilities :

- Clinical picture.
- Medical imaging.
- Immunological diagnosis (serology):
  - indirect immunofluorescence (IFAT) [screening]
  - Enzyme ImmunoAssay (EIA) [screening]
  - Indirect hemagglutination (IHA) [screening]
  - confirmation-test: immunoblot (arc 5 in double diffusion)
- (Identification of protoscolices and/or typical hooks in punction-fluid. The punction-fluid is clear; the hydatic sand can contain many protoscolices and/or liberated hooks.)



Morphology of the (proto)scolices and hooklets :

Dimensions : protoscolex: ±170 µm x ±110 µm
hooks: ±20 µm

Aspect protoscolex: oval to round

Contents protoscolex: large number of hooks are clearly visible

Colour protoscolex: colourless, transparent

Associated biological signs :

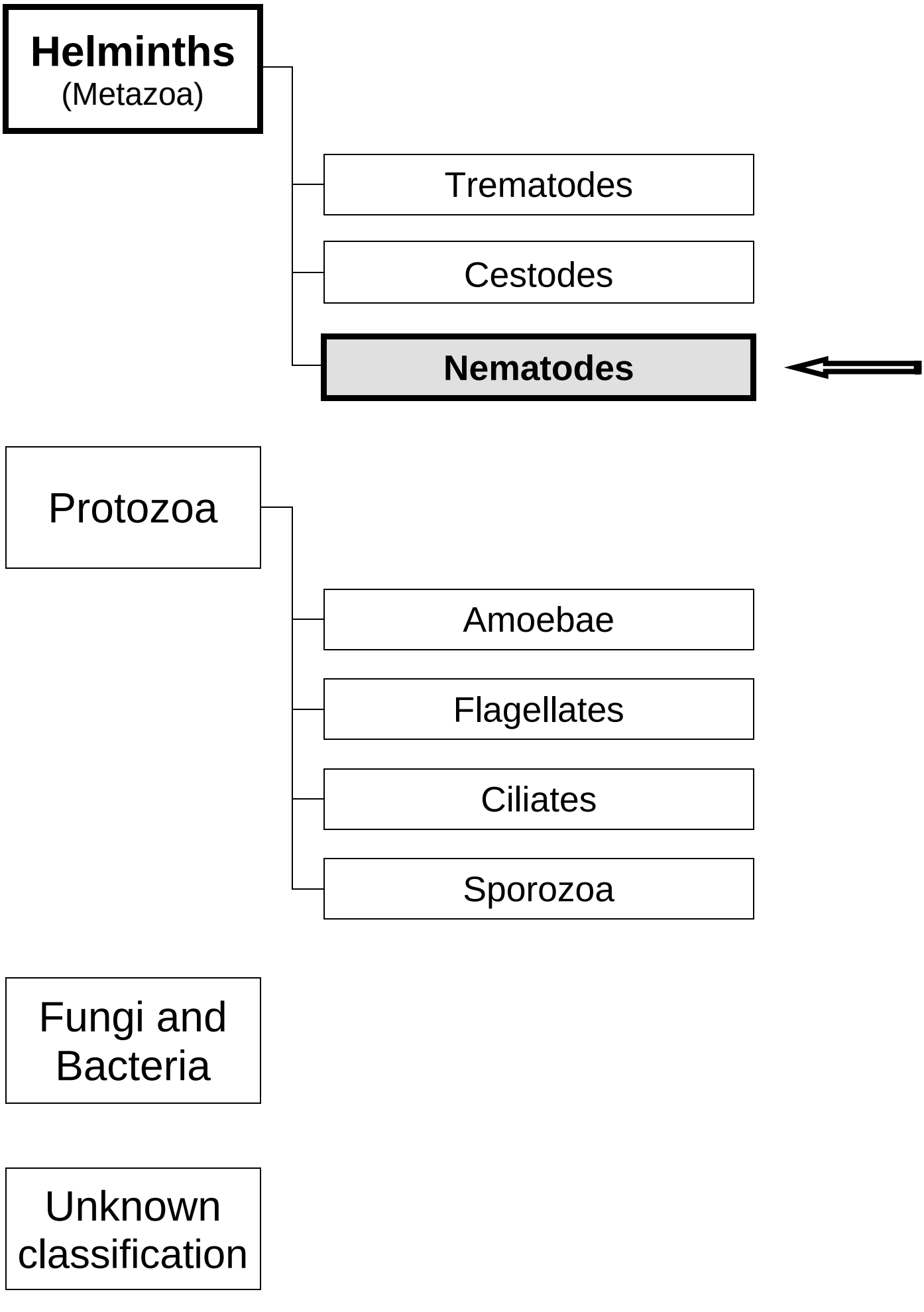
- Strong hypereosinophilia (especially in early stage)

Possible confusion with :

- other echinococcoses

Remarks :

- **Punction of hydatic cysts is NOT INDICATED.** By doing this, hydatic fluid with liberated protoscolices can disseminate and cause new hydatic cysts elsewhere in the body. Punction of hydatic cysts can also cause anaphylactic shock.
- Eggs of *Echinococcus* spp. are never found in human stools (eggs of *Echinococcus* spp. are morphologically identical to those of *Taenia* spp.).
- Hydatic cysts of *E. multilocularis* usually do not contain protoscolices (sterile cysts).
- Negative results in serology do not exclude echinococcosis (sensitivity of about 90%): indetectable antibodies, depending on the localisation, the integrity and the viability of the larvae. The sensitivity of serology is best in cases of hepatic or osseous localisations. Patients with calcified, dead or degenerating cysts are often seronegative.
- Serology for echinococcosis can be falsly positive in cases of infestations by other helminths, in cases of cancer and in cases of chronical immunological disorders. 5 to 25% of neurocysticercosis cases (*Taenia solium*) give positive serological reactions for echinococcosis. The same counts for cases of infestation with *Echinococcus multilocularis, Echinococcus vogeli* and *Echinococcus oligarthrus*.
- Other echinococcoses with almost identical life-cycles can (in rare cases) infest humans:
  - *E. multilocularis* (alveolar echinococcosis; northern hemisphere; fox tapeworm, usually with rodents as intermediate host (sometimes humans: 8 cases in Belgium between 1998 and 2005); serious pathology, usually hepatic localisation, more exceptionally pulmonary, renal or cerebral localisation).
  - *E. vogeli* (poly-cystic echinococcosis; Central- and South-America; tapeworm of wild dogs; intermediate host usually rodents (sometimes humans); usually hepatic or pulmonary localisations).
  - *E. oligarthrus* (extremely rare echinococcosis; Central- and South-America; cat tapeworm, usually rodents as intermediate host (sometimes humans); variable localisations possible).


Helminths
(Metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Ascaris lumbricoides

Superfamily :	**Class :**
Ascaridoidea	Nematodes

Geographic distribution :
Cosmopolitic

Common name :
Roundworm

Disease :
- NL : spoelworminfectie
- FR : ascaridiase
- EN : giant roundworm infection, ascariasis
- ES : ascaridiasis

Final host :
- Man
- Pigs
- Other animals ?

Intermediate host:
Without intermediate host and without vector

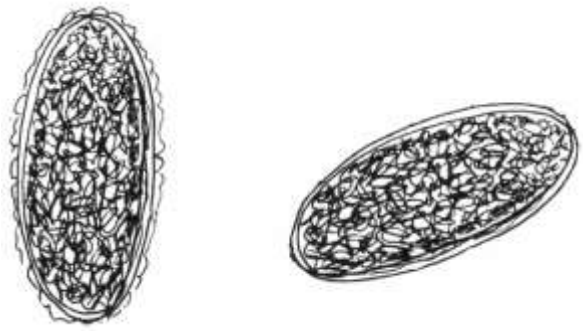
Transmission :
Oral ingestion of ***embryonated eggs***

Localisation of the adult worm :
In lumen of small intestine

Diagnostic possibilities :
- Searching for eggs in faeces, using: - Direct examination
  As the number of produced eggs usually is very high, concentration-techniques usually are unnecessary.
- Macroscopic identification of adult worms in vomit or faeces.
- Radiological examination can reveal infestations with only male worms.
- Serology (detection of specific Ab) will be positive during the phase of larval migration or in cases of infestation with only male worms.

Morphology of the fertile eggs:

Dimensions :	45-84 µm x 35-58 µm
Aspect :	oval or round
Shell :	Thick, smooth wall, usually with bumpy aspect, caused by albumine-coating
Contents :	usually one single round and granular cell (zygote)
Colour :	brown-yellow (when the albumine-coating is absent, the colour will be lighter and the egg will be more transparent)



Morphology of the non-fertile eggs:

Dimensions :	78-105 µm x 38-55 µm
Aspect :	oval, sometimes irregular shape
Shell :	Thin, smooth wall, usually with bumpy aspect, caused by albumine-coating
Contents :	homogenous, granular mass
Colour :	brown-yellow (when the albumine-coating is absent, the colour will be lighter and the egg will be more transparent)

Associated biological signs :
- Strong hypereosinophilia during migration-phase, moderate hypereosinophilia later on (Lavier's curve)
- Hyperleucocytosis

Possible confusion with :
- *Ascaris suum* (pig's roundworm)
- Fasciolidae
- *Schistosoma japonicum/S. mekongi*
- Plant cells and spores
- Pollen grains of saffran (*Carthamus tinctorius*),…
- …

Remarks :
- When body temperature is fluctuating (fever, death) or in case of stress (e.g. medication) the adult worms (usually the male ones) leave the body of the host via anus, mouth or nose.
- *A. suum* can infest as well pig as man, but will only rarely become adult in man (usually causes visceral *larva migrans*). Research suggests however that *A. suum* and *A. lumbricoides* could be the same species.
- Considering its great morphological variability and its strong resemblance to a variety of vegetal structures, the egg of *Ascaris* spp. is considered to be the most difficult to identify.
- Sometimes the larvae of *Ascaris* spp. can be found in the sputum (migration of larvae). Examination of sputum however is not the best technique for diagnosis.
- The eggs of *A. lumbricoides* need a certain period of time for "*maturation*" in the external environment before becoming infectious. This period of time depends on climatological factors (temperature, humidity,...) but is at least 18 days. The eggs can remain infectious for up to 5 years.
- The adult's lifespan is estimated at about 1 year, sometimes up to 3 years. The production of eggs starts about 2 to 3 months after initial infestation. One single female can contain up to 27 million eggs and produces up to 200.000 eggs per day.

Toxocara canis

SuperFamily : Ascaridoidea

Class : Nematodes

Geographic distribution :

Cosmopolitic

Common name :

Dog roundworm

Disease :

- NL : viscerale of oculaire *larva migrans*, toxocarose
- FR : *larva migrans* viscérale ou oculaire, toxocarose
- EN : visceral or ocular *larva migrans,* toxocariasis
- ES : *larva migrans* visceral o ocular, toxocariasis

Final host :

- Dogs
- Man (no development to adult worms)
  → paratenic visceral *larva migrans*

Intermediate host:

Without intermediate host and without vector

Transmission :

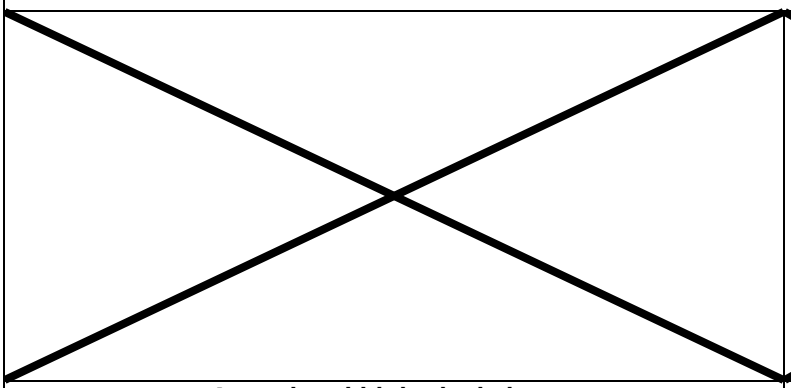
Oral ingestion of ***embryonated eggs***

Localisation of the larvae :

Blood and tissues (no development to adult worms)

Diagnostic possibilities :

- Clinical picture
- Serology: searching for specific antibodies in blood
- (Finding larvae in the eye, using an ophthalmoscope → accidental diagnosis)



Morphology of the eggs :

Dimensions :

Aspect :

Shell :

Contents :

Colour :

Associated biological signs :

- Hypereosinophilia

Possible confusion with :

- Other migrating larvae (*Ancylostoma* spp.*, Anisakis* spp.,...)
- *Larva currens* (*Strongyloides stercoralis)*

Remarks :

- **As the larvae do not develop to adult worms in human infestations, eggs are never found in human faeces.** If other eggs (e.g. those of *Ascaris lumbricoides, Trichuris trichiura,...*) are found in a patient's faeces, this indicates exposure to faecal material, increasing the probability of visceral *larva migrans* in case of clinical suspicion.
- *Toxocara cati* (cat roundworm) and *Baylisascaris procyonis* (racoon roundworm) also can cause *larva migrans* in humans, however more exceptional.
- Serology for *Toxocara* spp. is used only to confirm clinical suspicion (due to cross-reactivity). Serological tests, based on TES (Toxocara Excretory-Secretory antigens) offer high specificity, but cross-reactions with other nematodes are not rare. Evaluation of sensitivity and specificity of these serological tests continues to be hard due to the lack of parasitological methodes to confirm *Toxocara*-infestations (absence of a *Golden Standard*).

Anisakis spp.

SuperFamily : Ascaridoidea

Class : Nematodes

Geographic distribution :

Cosmopolitic
(consumption of raw fish)

Common name :

Herringworm

Disease :

- NL : anisakiase
- FR : anisakiase, anisakidose larvaire
- EN : anisakiasis, herringworm disease
- ES : anisakiasis

Final host :

- Aquatic mammals (seal,...)

Intermediate host :

1st: Crustaceans

2nd: Saltwaterfish (**Man** →paratenic)

Transmission :

By ingestion of fish, contaminated with ***living*** L3-larvae (macroscopically visible) (herring, sardine, mackerel,...)

Localisation of the larvae :

Parasitological deadlock (unless the larvae are excreted with the faeces).

Diagnostic possibilities :

- Clinical picture
- Serology: searching for specific antibodies in blood
- (Finding the larvae in tissues (endoscopy, gastroscopy, biopsy) → accidental diagnosis)

Morphology of the eggs :

Dimensions :

Aspect :

Shell :

Contents :

Colour :

Associated biological signs :

- Hypereosinophilia

Possible confusion with :

- Other migrating larvae (*Ancylostoma* spp.*, Toxocara* spp.,...)
- *Larva currens* (*Strongyloides stercoralis)*

Remarks :

- **As the larvae do not develop to adult worms in human infestations, eggs are never found in human faeces (paratenic host).**
- Serology for anisakiasis is not very sensitive, however it is still the most important means of diagnosis.
- Larvae of *Anisakis* spp. can survive low temperatures (-5°C) also pickling and smoking (up to 50°C) does not kill the larvae!!!
- Freezing at -20°C during at least 24h does kill the larvae.
- *Anisakis simplex* and *Anisakis marina* are only sporadically found in the Netherlands, as they systematically and sufficiently freeze herring.
- *Contracoecum* spp.*, Phocanema* spp. and *Pseudoterranova* spp.*,* also causing anisakiasis, are still regularly found in Japan, but also in the United States of America, in Chili, in the United Kingdom, in Germany and in Belgium.

Enterobius vermicularis

SuperFamily :	**Class :**
Oxyuroidea	Nematodes

Geographic distribution :

Cosmopolitic, however more frequently in temperate climates

Common name :

Pinworm

Disease :

- NL : oxyuriase
- FR : oxyurose
- EN : pinworm infection, enterobiasis
- ES : enterobiasis, oxiuriasis humana

Final host :

- Man

Intermediate host:

~~Without intermediate host and without vector~~

Transmission :

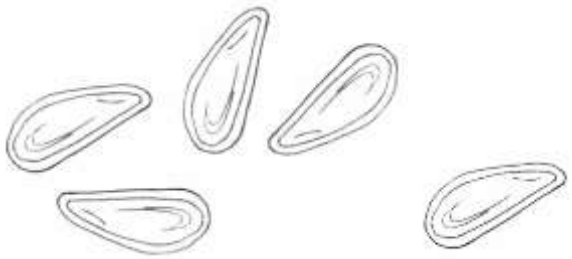
Oral ingestion of ***embryonated eggs***

Localisation of the adult worm :

In lumen of caecum and adjacent parts.

Diagnostic possibilities :

- Searching for eggs, using the tape-test.
- Finding the adult females on the peri-anal skin (usually at night).
- (Finding the eggs in a faecal sample (in direct examination or after concentration by sedimentation). Examination of faeces, searching for eggs is not very efficient.)



Morphology of the eggs :

Dimensions : 50-60 µm x 20-32 µm

Aspect : asymmetrically oval

Shell : double and smooth

Contents : embryo present (granular mass or larva)

Colour : colourless and transparent

Associated biological signs :

- Moderate, slightly oscillating hypereosinophilia

Possible confusion with :

- Adult females can be confused with food-remnants (plant-fibers etc.), pieces of toilet-paper,…
- Air-bulbs in a tape-test can be confused with eggs

Remarks :

- Adult females of *E. vermicularis* have a length of about 8 to 15 mm.
- A tape-test can also reveal eggs of *Taenia* spp.
- Sometimes the eggs can be found in the urine-sediment of little girls (vaginal ectopical localisation).
- Auto-infestation is quite frequent (especially in children and psychiatric patients).
- Hyper-infestation can occur through auto-infestation, as well as through retro-infestation. In this case, some larvae hatch from the eggs adhering to the peri-anal skin and migrate through the anus to develop into adults.
- As the eggs are placed on the peri-anal skin, they usually aren't found in the faeces.
- The tape-test sometimes is called Scotch-Test or Graham's Test.
- The tape-test is best performed in the morning before making one's toilet and before defecation.
- If the tape-test is performed 3 times in one week, one can reach a sensitivity of 95%.
- *E. grigorii* has been described in Europe, Asia and Africa, but its morphology, its life-cycle, the clinical aspect and the treatment of *E. grigorii* are identical to that of *E. vermicularis*. The difference between both can only be made by using molecular techniques (PCR).
- Development into adult takes about 3 weeks time and does not include any migration-phase.
- The adult's lifespan is estimated at less than 55 days.
- One single female can contain up to 10.000 eggs and produces about 500 eggs per day.

Trichuris trichiura

Superfamily : Trichuroidea

Class : Nematodes

Geographic distribution :

Cosmopolitic, more frequent in tropical and sub-tropical countries.

Common name :

Whipworm

Disease :

- NL : trichuriasis, zweepworm infectie
- FR : trichocéphalose, trichuriase
- EN : whipworm infection, trichuriasis
- ES : tricocefalosis

Final host :

- Man

Intermediate host:

~~Without intermediate host and without vector~~

Transmission :

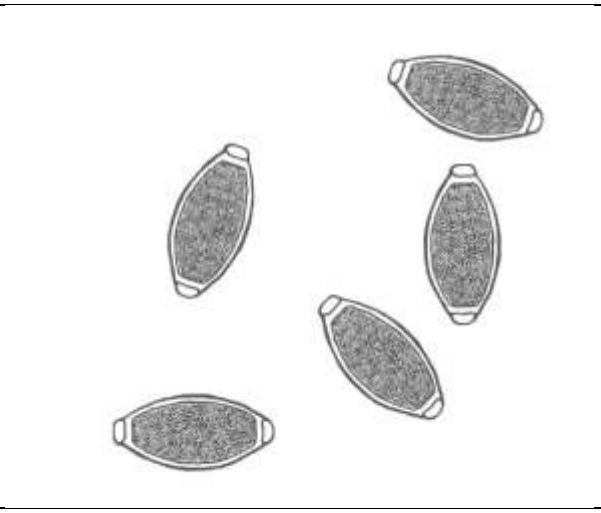
Oral ingestion of ***embryonated eggs***

Localisation of the adult worm :

Intestine
(in mucosa of caecum, appendix and large intestine)

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
  - Concentration by sedimentation
  - Concentration by flottation
- Macroscopic: Sometimes the very typical adult worms can be found on/in the faeces.



Morphology of the eggs :

Dimensions : 49-65 µm x 20-29 µm

Aspect : lemon-shaped

Shell : thick, smooth, brown-yellow wall

Contents : homogenous, granular mass

Colour : orange-brown

Characteristics : two plugs; colourless, transparent and rounded polar knobs

Associated biological signs :

- Hypereosinophilia (± 15%)
- Especially in children, a high wormload can cause a ferriprivic, hypochromic anaemia

Possible confusion with :

- Eggs of *Capillaria* spp.
- *Trichuris vulpis*
- Pollen grains (Iris)

Remarks :

- The adult worms, measuring between 3 en 5 cm, are typically "whip"-shaped (the anterior part is thin and filamentous, while the posterior part is thick).
- Sometimes eggs can be found with abnormal aspect and/or abnormal thickness of the wall and/or abnormal colour etc. This phenomenon is usually caused by anti-helminthics or confusion with eggs of *T. vulpis* (*Trichuris* of dogs→ passing eggs).
- The eggs of *T. trichiura* need a certain period of time for "*maturation*" in the external environment before becoming infectious. This period of time depends on climatological factors (temperature, humidity,...) but lies between 10 days and several months.
- Counting the number of eggs (e.g. expressed in eggs per gram (EPG)) can be useful, because the pathology often depends on the degree of infestation.
- Some persons which are sensitive to this infection, already develop a pathology at low degrees of infestation.
- Development into adult entirely takes place in the intestinal lumen, not incuding any migration-phase.
- The adult's lifespan is estimated at 1 to 4 years (some sources mention lifespans up to 20 years).
- Eggs appear in faeces about 30 to 90 days after initial infestation.
- Eggs produced per female: 3.000 to 20.000 eggs per day.

Capillaria hepatica

SuperFamily : Trichuroidea

Class : Nematodes

Geographic distribution :

Cosmopolitic.

Common name :

Disease :

- NL : lever-capillariase
- FR : capillariose hépatique
- EN : hepatic capillariasis
- ES : capillariasis hepatica

Final host :

- Rodents
- Other animals
- Sometimes man (zoonosis)

Intermediate host :

None
(carnivore only serves to **disperse** the eggs)

Transmission :

Oral ingestion of ***embryonated eggs***

Localisation of the adult worm :

Parenchymal cells of the liver.

Diagnostic possibilities :

- Detection of eggs (or worms) in biopsy of the liver (in case of liver-infestation).

Morphology of the eggs :

Dimensions : 50-60 µm x 20-30 µm

Aspect : oval

Shell : Thick with usually a dense radial structure

Contents : homogenous, granular mass

Colour : dark brown-grey

Characteristics : two plugs; colourless, transparent polar knobs which stay **inside the shell**

Associated biological signs :

- hypereosinophilia
- Disturbed liver tests
- Anemia
- Hyperleukocytosis

Possible confusion with :

- Eggs of *Trichuris trichiura*
- Eggs of *Trichuris vulpis*
- Eggs of other *Capillaria* spp.

Remarks :

- The eggs of the different *Capillaria* spp. are morphologically identical: when one of these eggs are found, the most correct answer should be: *"Eggs of Capillaria spp. found"*.
- *Capillaria hepatica* is also known as *Hepaticola hepatica or Calodium hepaticum*.
- Eggs of *C. hepatica* which have been found in a faecal sample, are usually passing eggs (consumption of liver, infested with adult worms). After their passing, these eggs will develop outside the host's body into embryonated and infectious eggs. In this case, man serves as a carnivore to *disperse* the eggs.
- In cases of liver-infestation, the produced eggs will remain stuck in the host's liver. They will only get to the outer environment when the host dies (desintegration of the host's body) or when the host is consumed by a carnivore, causing the eggs to be passed in the external environment with the carnivore's faeces.
- The eggs of *C. hepatica* need a certain period of time for "*maturation*" in the external environment before becoming infectious. This period of time depends on climatological factors (temperature, humidity,...)
- Eggs appear in faeces about 2 to 3 weeks after initial infestation.
- The adult's lifespan is estimated at 1 to 4 months.
- Up to 2005, only about 100 cases have been reported in literature.

Capillaria philippinensis

SuperFamily : Trichuroidea

Class : Nematodes

Geographic distribution :

Endemic in the Philippines and Thaïland. Rare in Asia, Colombia and the Middle-East.

Common name :

Disease :

- NL : intestinale capillariase
- FR : capillariose intestinale
- EN : intestinal capillariasis
- ES : capillariasis intestinal

Final host :

- Fish-eating birds
- Sometimes man (zoonosis)

Intermediate host :

Freshwater-fish

Transmission :

Consumption of fish, infested with living larvae.

Localisation of the adult worm :

Lumen of small intestine.

Diagnostic possibilities :

- Detection of the eggs in faeces, using: - direct examination (zie p.102)
  - concentration by sedimentation (zie p.105)
- Macroscopically finding the adult worms in/on the faeces: ♂ : 2,3 – 3,2 mm
  ♀ : 2,5 – 4,3 mm

Morphology of the eggs :

Dimensions : 36-45 µm x 20-25 µm

Aspect : oval

Shell : Thick with usually a dense radial structure

Contents : homogenous, granular mass

Colour : dark brown-grey

Characteristics : two plugs; colourless, transparent polar knobs which stay **inside the shell**

Associated biological signs :

- Hypereosinophilia

Possible confusion with :

- Eggs of *Trichuris trichiura*
- Eggs of *Trichuris vulpis*
- Eggs of other *Capillaria* spp.

Remarks :

- *Capillaria philippinensis* is also known as *Paracapillaria philippinensis, Calodium philippinensis* or *Aonchotheca philippinensis*.
- The eggs of the different *Capillaria* spp. are morphologically identical: when one of these eggs are found, the most correct answer should be: *"Eggs of Capillaria spp. found"*.
- The adult females produce non-embryonated eggs in the intestinal lumen. Some of these eggs can however already embryonate inside the intestines, liberating infectious larvae. These larvae can cause an auto-infestation, resulting in very high parasitaemias.
- As the shedding of the eggs of *Capillaria philippinensis* is intermittent, examination of multiple stool-samples can be necessary to make the diagnosis.

Capillaria aerophila

SuperFamily : Trichuroidea

Class : Nematodes

Geographic distribution :

Cosmopolitic.

Common name :

Disease :

- NL : pulmonaire capillariase
- FR : capillariose pulmonaire
- EN : pulmonary capillariasis
- ES : capillariasis pulmonar

Final host :

- dogs, cat, fox, ...
- Sometimes man (zoonosis)

Intermediate host :

None

(earthworm??? → only serves to **disperse** the eggs**?**)

Transmission :

Oral ingestion of ***embryonated eggs***

Localisation of the adult worm :

Lumen of the trachea and bronchia.

Diagnostic possibilities :

- Detection of the eggs in faeces, using:
  - direct examination (zie p.102)
  - concentration by sedimentation (zie p.105)
- Identification of adult worms in biopsy of the lungs.
- (Identification of the eggs in sputum.)

Morphology of the eggs :

Dimensions : 50-60 µm x 20-30 µm

Aspect : oval

Shell : Thick with usually a dense radial structure

Contents : homogenous, granular mass

Colour : dark brown-grey

Characteristics : two plugs; colourless, transparent polar knobs which stay **inside the shell**

Associated biological signs :

- Hypereosinophilia

Possible confusion with :

- Eggs of *Trichuris trichiura*
- Eggs of *Trichuris vulpis*
- Eggs of other *Capillaria* spp.

Remarks :

- *Capillaria aerophila* is also known as *Eocoleus aerophilus*.
- The eggs of the different *Capillaria* spp. are morphologically identical: when one of these eggs are found, the most correct answer should be: *"Eggs of Capillaria spp. found"*.
- The eggs of *C. aerophila* need a certain period of time for "*maturation*" in the external environment before becoming infectious. This period of time depends on climatological factors (temperature, humidity,...)
- The adult's lifespan is estimated at about 1 year.
- Up to 2005, less than 100 cases have been reported in literature.

Trichinella spp.

SuperFamily : Trichuroidea

Class : Nematodes

Geographic distribution :

Cosmopolitic
(except in Australia)

Common name :

Trichine

Disease :

- NL : trichinose
- FR : trichinose, trichinellose
- EN : trichinosis, trichinellosis
- ES : triquinosis

Final host :

- Mammals
- (Man)

Intermediate host:

Without intermediate host
and
without vector

Transmission :

Ingestion of meat, infested with living larvae.

Localisation of the adult worm :

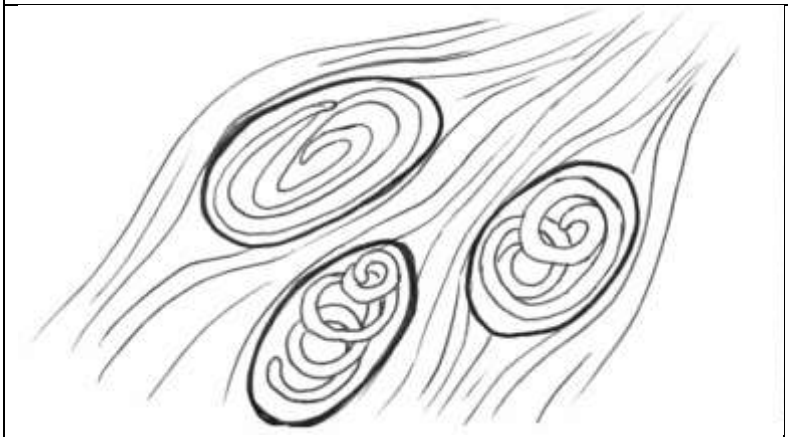
Mucosa of the small intestine

Localisatie larve :

Encapsulated in striated muscles

Diagnostic possibilities :

- (Suggestive) clinical picture.
- Serology (detection of specific Ab against ES-Ag)
- Detection of typical cysts in biosies of striated muscle-tissue.
  The biopsy is pressed between two glass slides and examined at low magnification (40x or 100x total magnification). Staining is not necessary.
  Concentration can be useful (see p.114).
- (Identification of adult worms or larvae in faeces)



Morphology of the cysts :

Dimensions : 400-500 µm x 250 µm

Aspect : oval to lemon-shaped,
parallel with muscular fibres

Contents : rolled-up larva

Characteristics :
calcification possible

Associated biological signs :

- Hypereosinophilia (especially in migrational phase)
- Elevated muscular enzymes in serum (CK, LDH, aldolase)

Possible confusion with :

- Other *Trichinella* spp.

Remarks :

- Muscular trichines are killed by:
  - pickling: during at least 6 weeks in 19% NaCl
  - heat: frying, coocking, etc. (during at least 10 minutes at 80°C)
  - cold: during at least 10 days at -18°C
- The adult worm (1-4 mm x 60-75 µm) or the larvae (80-120 µm) can exceptionally be found in the faeces. The adult's life span in the intestinal mucosa is limited (maximum 4 months). During this period the female worms produce 1000 to 2000 larvae which migrate to the striated muscles and encapsulate by "nurse cell"-forming, actively modifying the host cell to their proper needs and transforming the cellular membrane.
- *T. spiralis* (cosmopolitic, mammals and omnivores) is usually responsible for infestations of man. Other *Trichinella* spp. are however also found in man: *T. pseudospiralis* (cosmopolitic, mammals and birds), *T. nativa* (polar bears), *T. nelsoni* (african predators), *T. britovi* (carnivores in Europe and Western asia), ...
- The preferred serological tests are those based on TSL-1 ES-Ag as this Ag is found in all different *Trichinella* spp.
- Serology usually becomes positive 3 to 5 weekes after initial infestation.

Strongyloides stercoralis

SuperFamily : Rhabdiasoidea

Class : Nematodes

Geographic distribution :

Tropical and sub-tropical countries.
Also in temperate climates in certain circumstances (mining, tunnel-construction,…)

Common name :

Threadworm

Disease :

- NL : strongyloidosis
- FR : anguillulose
- EN : threadworm infection
  strongyloidiasis
- ES : estrongiloidiasis
  anguillulosis

Final host :

- Man
- Chimpansee
- Dog
- Cat

Intermediate host:

Without intermediate host
and
without vector

Transmission :

- In case of contact with ground or mud contaminated with infectious larvae, these larvae will penetrate the skin or the mucosa.
- Auto-infestation: trans-intestinal or trans-peri-anal

Localisation of the adult worm :

In mucosa of duodenum.

Diagnostic possibilities :

- Searching for eggs in faeces, using: - Direct examination
  - Concentration by sedimentation
- Sarching for rhabditiform (or filariform) larvae, using: - Direct examination
  - Concentration by sedimentation
  - **Baermann concentrationtechnique**
  - Agar-culture
- Serology (detection of specific Ab (ELISA) using Ag of filariform larvae)
- (Identification of the larvae in duodenal aspirate or in string-test (for *Giardia lamblia*))

Morphology :

The morphological aspects of the different stages have been brought together in a schematic overview.
In this overview, also the differential diagnosis with Hookworms is given (see p.47).

Morphology of the rhabditiform larvae :

Dimensions : 200-300 µm x 16-20 µm

Mouth cavity : shallow (⅓ to ½ of the width of the larva)

Bulbus : present

Tail : pointed

Associated biological signs :

- Oscillating hypereosinophilia (going from 5% to 30%)
- Often presence of Charcot-Leyden crystals in faeces

Possible confusion with :

- Vegetal fibers and hairs
- Eggs and larvae of Hookworms
- Eggs of *Trichostrongylus* spp.
- Eggs of *Ascaris* spp. (without albumine coating)
- Eggs of *Enterobius vermicularis*
- Eggs of *Acarina* spp. (mite)

Remarks :

- Strongyloidosis is a disease which can evolve quite seriously in case of immuno-suppression.
- The eggs of *S. stercoralis* usually already hatch inside the intestins, meaning that usually the **rhabditiform larvae** will be found in (fresh) faeces. Filariform larvae will only be found in faeces of at least several hours old or in case of auto-infestations.
- Eggs are only sporadically found in cases of serious diarrhoea and usually will contain an embryo.
- The adult males of Strongyloides stercoralis measure about 1 to 2 mm, while adult females reach up to 2 to 3 mm. The adult's lifespan is rather limited, but the possibility of auto-infestation makes that an infestation with *S. stercoralis* can continue for as long as 30 years after leaving an endemic area. In cases of trans-peri-anal auto-infestation, the larvae, passing sub-cutaneously, will cause a typical *larva migrans*, called *larva currens.*
- Sometimes the larvae of *S. stercoralis* are found in a patient's sputum (migration of larvae). Examination of sputum however is not the best technique for diagnosis.
- Other *Strongyloides* spp. (*S. fuelleborni*) more common in chimpansees and baboons, can occur sporadically in humans, but with limited pathology. In these cases, usually eggs (with blastomeres) are found in (fresh) faeces.
- All other *Strongyloides* spp. (which usually occur in animals) can only develop to cutaneous *larva migrans* when infesting humans.
- Serology has a sensitivity of about 90%. When successfully treated, titers will drop significantly after 9 to 12 months.
- The larvae can be found in the patient's faeces about 2 to 3 weeks after initial infestation.

Ancylostoma duodenale

SuperFamily : Ancylostomatoidea

Class : Nematodes

Geographic distribution :

- Europe
- Northern-Africa
- Asia

Common name :

Hookworm

Disease :

- NL : mijnwormziekte
  ancylostomiasis
- FR : ankylostomiase
- EN : Old World hookworm infection
  ancylostomiasis
- ES : uncinariasis
  anaemia de los mineros

Final host :

- Man
- Pig
- Felidea, canidea
- Some monkey-species

Intermediate host:

Without intermediate host
and
without vector

Transmission :

In case of contact with ground or mud contaminated with infectious larvae, these larvae will penetrate the skin (or possibly the mucosa).

Localisation of the adult worm :

Attached to jejunum and duodenum.

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
  - Concentration by sedimentation
  - Concentration by flottation

  Sometimes (when the faeces cannot be examined immediately: >12h) the larvae (instead of the eggs) can be found in the faeces.

Morphology :

The morphological aspects of the different stages have been brought together in a schematic overview.
In this overview, also the differential diagnosis with *S. stercoralis* is given (see p.47).

Morphology of the eggs :

Dimensions :	60-80 µm x 35-40 µm
Aspect :	oval
Shell :	very thin and fine
Contents :	blastomeres (usually 4) in freshly produced faeces
Colour :	colourless and transparent with brown-greyish blastomeres

Associated biological signs :

- Hypereosinophilia (± 30%)
- Hypochromic, microcytic anaemia can occur in case of chronic infestation.
- Drop of iron in serum in case of chronic infestation

Possible confusion with :

- Vegetal fibers and hairs
- Eggs and larvae of *Strongyloides stercoralis*
- Eggs and larvae of *Necator americanus*
- Eggs of *Trichostrongylus* spp.
- Eggs of *Ternidens deminutus*
- Eggs of *Ascaris* spp. (without albumine coating)
- Eggs of *Acarina* spp. (mite)
- Eggs of *Heterodera* spp. (plant nematodes)

Remarks :

- The optimal temperature for the development of *A. duodenale* is about 21-27°C.
- As the eggs (and larvae) of *Ancylostoma duodenale, Ancylostoma ceylanicum* and *Necator americanus* are morphologically indistinguishable using an ordinary microscope, they are reffered to (in microscopic examinations) as “eggs (or larvae) of hookworms”.
- One should always be aware that in most cases the **eggs** (usually containing 4 blastomeres) are found in (fresh) faeces. Larvae are only found in faeces of more than 12h old.
- As a result of importation of parasites, geographic distribution is no longer a useful criterium for differentiation of *Ancylostoma duodenale* and *Necator americanus*.
- The consumption (and spill) of blood for *Ancylostoma duodenale* is estimated at about 0,2 ml per day per worm (0,02 ml for *Necator americanus*).
- Sometimes the larvae of *A. duodenale* are found in a patient’s sputum (migrating larvae). Examination of sputum however is not the best technique for diagnosis.
- Other *Ancylostoma* spp. (which usually infest animals) can also infest humans. *A. ceylanicum* (hamsters) is morphologically and pathologically identical to *A. duodenale*. *A. caninum* (dog) and *A. braziliense* (cat) can only develop to cutaneous *larva migrans* when infesting humans.
- The eggs of *Oesophagostomum* spp. (a zoonotic parasite which can be found in Africa, Indonesia and Brasil and which causes “(multi-)nodular disease”) are morphologically identical to those of Hookworms.
- The eggs of *Ternidens deminutus* closely resemble hookworm eggs, but are remarkably larger (80-85 µm x 40-55 µm).
- The adult’s lifespan is estimated at 1 to 9 years. It’s length is about 0,5 to 1 cm for males and 1 to 2 cm for females.
- Eggs appear in faeces about 15 to 40 days after initial infestation.
- Eggs produced per female: 5.000 to 22.000 eggs per day.

Necator americanus

SuperFamily : Ancylostomatoidea

Class : Nematodes

Geographic distribution :

- America
- Africa
- China
- Indonesia
- Australia

Common name :

Hookworm

Disease :

- NL : mijnwormziekte
  ancylostomiasis
- FR : ankylostomiase
- EN : New World hookworm infection
  ancylostomiasis
- ES : uncinariasis
  anaemia de los mineros

Final host :

- Man

Intermediate host:

Without intermediate host and without vector

Transmission :

In case of contact with ground or mud contaminated with infectious larvae, these larvae will penetrate the skin.

Localisation of the adult worm :

Attached to jejunum and duodenum.

Diagnostic possibilities :

- Searching for eggs in faeces, using:
  - Direct examination
  - Concentration by sedimentation
  - Concentration by flottation

  Sometimes (when the faeces cannot be examined immediately: >12h) the larvae (instead of the eggs) can be found in the faeces.

Morphology :

The morphological aspects of the different stages have been brought together in a schematic overview.
In this overview, also the differential diagnosis with *S.stercoralis* is given (see p.47).

Morphology of the eggs :

Dimensions :	60-80 µm x 35-40 µm
Aspect :	oval
Shell :	very thin and fine
Contents :	blastomeres (usually 8) in freshly produced faeces
Colour :	colourless and transparent with brown-greyish blastomeres

Associated biological signs :

- Hypereosinophilia (± 30%)
- (Hypochromic, microcytic anaemia can occur in case of chronic infestation)
- (Drop of iron in serum in case of chronic infestation)

Possible confusion with :

- Vegetal fibers and hairs
- Eggs and larvae of *Strongyloides stercoralis*
- Eggs and larvae of *Ancylostoma* spp.
- Eggs of *Trichostrongylus* spp.
- Eggs of *Ternidens deminutus*
- Eggs of *Ascaris* spp. (without albumine coating)
- Eggs of *Acarina* spp. (mite)
- Eggs of *Heterodera* spp. (plant nematodes)

Remarks :

- The optimal temperature for the development of *N. americanus* is about 25-35°C.
- As the eggs (and larvae) of *Ancylostoma duodenale, Ancylostoma ceylanicum* and *Necator americanus* are morphologically indistinguishable using an ordinary microscope, they are reffered to (in microscopic examinations) as “eggs (or larvae) of hookworms”.
- One should always be aware that in most cases the **eggs** (usually containing 8 blastomeres) are found in (fresh) faeces. Larvae are only found in faeces of more than 12h old.
- As a result of importation of parasites, geographic distribution is no longer a useful criterium for differentiation of *Ancylostoma duodenale* and *Necator americanus*.
- The consumption (and spill) of blood for *Necator americanus* is estimated at about 0,02 ml per day per worm (0,2 ml for *Ancylostoma duodenale*).
- Sometimes the larvae of *N. americanus* are found in a patient’s sputum (migrating larvae). Examination of sputum however is not the best technique for diagnosis.
- The eggs of *Oesophagostomum* spp. (a zoonotic parasite which can be found in Africa, Indonesia and Brasil and which causes “(multi-)nodular disease”) are morphologically identical to those of Hookworms.
- The eggs of *Ternidens deminutus* closely resemble hookworm eggs, but are remarkably larger (80-85 µm x 40-55 µm).
- The adult’s lifespan is estimated at 4 to 20 years. It’s length is about 0,5 to 1 cm for males and 1 to 2 cm for females.
- Eggs appear in faeces about 15 to 40 days after initial infestation.
- Eggs produced per female: 3.000 to 6.000 eggs per day.

Strongyloides stercoralis / Hookworms : overview

Strongyloides stercoralis

Egg containing larva :

50-76 µm

very rare

(usually 50-55 µm)

Rhabditiform larva :

< 1 hour

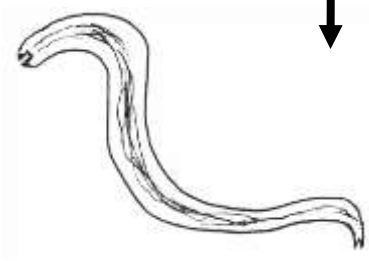
200-300 µm x 16-20 µm
(usually 225 x 18 µm)
Bulbus present
Shallow mouth-cavity
Pointed tail

Most common stage

Filariform larva :

several hours

500-550 µm x 20-24 µm
(usually 500 x 22 µm)
Bulbus absent
Shallow mouth-cavity
Bifurcated tail



Rare

Hookworms

Egg containing blastomeres :

60-80 µm
(usually 60-65 µm)

most common stage

Rhabditiform larva :

± 12 hours

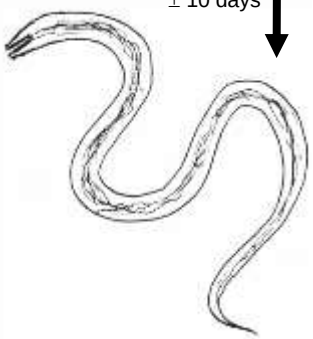
200-300 µm x 14-17 µm
(usually 250 x 16 µm)
Bulbus present
Deep mouth-cavity
(equal to diameter of larva)
Pointed tail

Rare

Filariform larva :

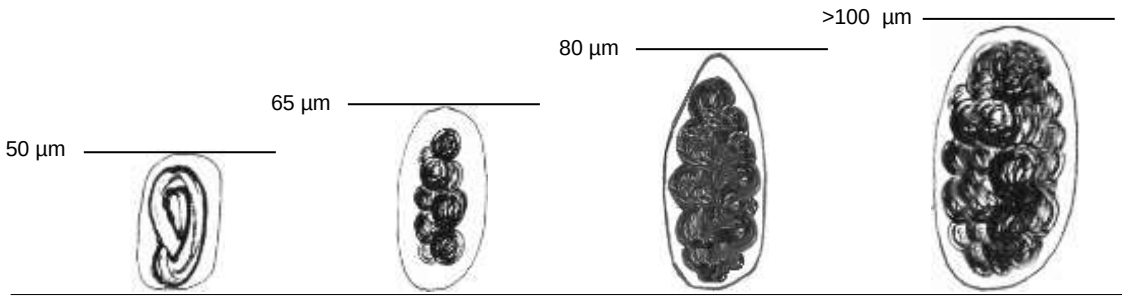
± 10 days

500-700 µm x 20-24 µm
(usually 700 x 22 µm)
Bulbus absent
Deep mouth-cavity
(equal to diameter of larva)
Pointed tail



Very rare

Comparison :



Egg of *Strongyloides stercoralis* Egg of hookworms Egg of *Trichostrongylus* spp. Egg of *Acarina* spp. (mite)

Trichostrongylus spp.

SuperFamily : Trichostrongyloidea

Class : Nematodes

Geographic distribution :

Cosmopolitic

Common name :

Disease :

- NL : trichostrongylosis
- FR : trichostrongylose
- EN : trichostrongyliasis
- ES : trichostrongiliasis

Final host :

- Ovidae, goat
- Bovidae
- Camel-like animals, horse, pig
- Sometimes man (zoönose)

Intermediate host:

Without intermediate host and without vector

Transmission :

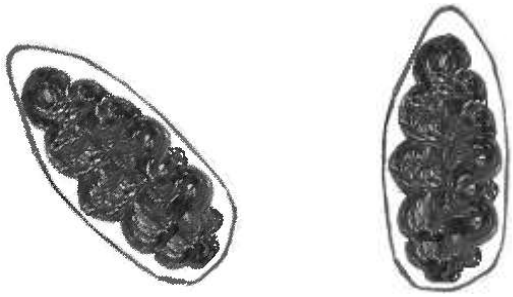
Oral ingestion of the filariform larvae.

Localisation of the adult worm :

Intestinal lumen

Diagnostic possibilities :

- Detection of the eggs in faeces, using :
  - Direct examination
  - Concentration by sedimentation
  - Concentration by flottation



Morphology of the eggs :

Dimensions : 79-100 µm x 40-50 µm

Aspect : egg-shaped; one pole more stretched out than the other

Shell : thin and smooth

Contents : usually a lot of blastomeres; sometimes a morula

Colour : colourless and transparent with grey to brown-grey blastomeres

Associated biological signs :

- Hypereosinophilia
- (Hypochromic, microcytic anaemia can occur in case of chronic infestation)
- (Drop of iron in serum in case of chronic infestation)

Possible confusion with :

- Eggs of *Strongyloides stercoralis*
- Eggs of *Necator americanus*
- Eggs of *Ancylostoma* spp.
- Eggs of *Ternidens deminutus*
- Eggs of *Ascaris* spp. (without albumine coating)
- Eggs of *Acarina* spp. (mite)

Remarks :

- After (oral) infestation the larvae do not migrate to the lungs. The larvae develop to adult worms inside the intestinal lumen.
- Up till now, 6 different species of *Trichostrongylus* are known to infect humans.
- The eggs of these species are morphologically identical and therefore best addressed to as “eggs of *Trichostrongylus* spp.”
- The eggs of *Ternidens deminutus* closely resemble *Trichostrongylus* eggs, but are rather oval instead of egg-shaped.
- Eggs appear in faeces about 3 to 5 weeks after initial infestation.
- All species of this superfamily, infecting man, are zoonoses. Some of them infest humans but sporadically, others are responsible for large scale infestations.

Onchocerca volvulus

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- West- and Central Africa
- Yemen
- Central America
- North-East Brasil
- North-East Venezuela

Common name : (Filaria)

Disease :

- NL : onchocercose (rivierblindheid)
- FR : onchocercose (cécité des rivières)
- EN : river blindness, onchocerciasis
- ES : oncocercosis

Final host :

- Man

Intermediate host:

(Vector)

Simulium spp.

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm :

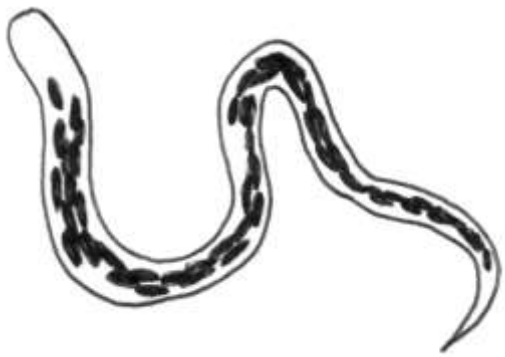
Sub-cutaneous tissues.
Free-living or sometimes multiple worms together, encapsulated in hard nodules (onchocercomes).

Localisation of the microfilaria :

Skin.

Diagnostic possibilities :

- Searching for microfilaria, using: - Skin-Snip (good screening-method)
  - Deep scarification
- Mazzotti's Test (this pathognomic test, using DEC, is not indicated for reasons of possible allergic reactions)
- Searching for adult worms in possibly present onchocercomes
  (Surgical intervention!!! Risk of allergic reaction!!!)
- Serology (searching for Ab (mostly IgE-type) in serum)
- Detection of specific DNA, using molecular techniques (PCR)
- Finding the microfilaria in the eye, using a split-lamp



Morphology of the microfilaria (Giemsa) :

Dimensions : 280-320 µm x 5-9 µm

Tail : Empty and pointed, usually curved

Head : long empty space (≈ 2x width of the larva)

Sheath : not present

Characteristics :
oval nuclei, usually not very clear

Associated biological signs :

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp., *Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- When performing a Mazzotti'sTest (using DEC, NOT using ivermectine) the microfilaria can sometimes be found in the blood and even in urine.
- *Onchocerca volvulus* is an aperiodical microfilaria.
- Deep scarifications always contain a small amount of blood, which explains the possibility of finding blood-microfilaria in such a preparation.
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.
- It takes at least 6 months for the larvae to become adult worms. The adult's lifespan is estimated at more than 15 years. The microfilaria can survive up to 2 years in the skin.

Loa Loa

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- Tropical rain-forests, mostly of Western- and Central Africa

Common name : (Filaria)

Disease :

- NL : loasis
- FR : loase
- EN : eye worm disease, loiasis
- ES : loasis

Final host :

- Man
- Primates

Intermediate host:

(Vector)

Horse-flies (*Chrysops* spp.)

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm :

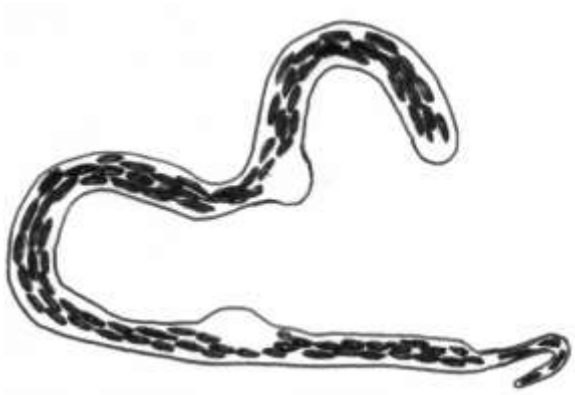
Sub-cutaneous tissues and conjunctiva.

Localisation of the microfilaria :

Blood.

Diagnostic possibilities :

- Examination of blood: Direct examination (not very efficient; no identification possible!)
  Thick blood smear: - Giemsa staining is a good technique, but the sheath usually doesn't take up the colour.
  - Carazzi staining is better, but more complex.
  Woo-technique: good concentration-technique (no identification possible!)
  KNOTT-method: very efficient concentration-method.
  After filtration onto a membrane, followed by staining (epidemiological purposes).
- Sometimes the adult worm (up to 7 cm long) passes under the conjunctiva of the eye ("accidental diagnosis").
- Serology (searching for Ab in serum)
- Detection of specific DNA, using molecular techniques (PCR)



Morphology of the microfilaria (Giemsa):

Dimensions : 230-250 µm x 5-7 µm

Tail : stretched, blunt tail, often folded

Head : small empty space

Sheath : present, but doesn't always stain with Giemsa.

Characteristics :
Oval shaped nuclei, filling the tail. Two hernia-like saggings at about 1/3 and 2/3 of its body-length.

Associated biological signs :

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp., *Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- The microfilaria mainly circulate during the day in the periferal bloodstream (bloodsample best taken between 11 and 16 o'clock).
- The presence of microfilaria in the blood can fluctuate a lot.
- The searching for the microfilaria themselves often yields negative results during the first years of infestation.
- Very rarely the microfilaria of *Loa Loa* can be found in urine-samples.
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.
- It takes 6 to 12 months for the larvae to become adult worms. The adult's lifespan is estimated at 4 to 7 years (up to 25 years according to certain authors).

Wuchereria bancrofti

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- All tropical and subtropical regions (except very dry regions)
- Turkey (rare)

Common name :

Bancroft's filaria

Disease :

- NL : wuchereriasis
- FR : wuchereriase
- EN : bancroftian filariasis, lymphatic filariasis
- ES : wuchereriasis, filariasis linfática

Final host :

- Man

Intermediate host:

(Vector)

Different kinds of mosquitoes: Family of *Culicidae* (*Culex* spp.*, Mansonia* spp.*, Anopheles* spp.*, Aedes* spp.*)*

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm :

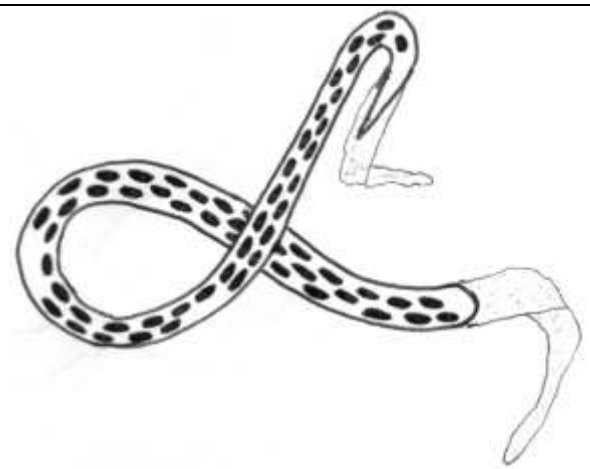
Lymphatic system.

Localisation of the microfilaria :

Blood.

Diagnostic possibilities :

- Examination of blood: Direct examination (not very efficient; no identification possible!)
  Thick blood smear: - Giemsa staining is a good technique, but the sheath usually doesn't take up the colour.
  - Carazzi staining is better, but more complex.
  Woo-technique: good concentration-technique (no identification possible!)
  KNOTT-method: very efficient concentration-method.
  After filtration onto a membrane, followed by staining (epidemiological purposes).
- Serology (searching for Ab (mostly IgG4-type) in serum)
- Searching for Ag in blood, using Dip-Stick (same sensitivity as KNOTT-method)
- Detection of specific DNA, using molecular techniques (PCR)
- Detection of adult worms and abnormal lymphatic dilatations, using medical imaging



Morphology of the microfilaria (Giemsa):

Dimensions : 240-300 µm x 7,5-10 µm

Tail : empty, pointed and often folded

Head : small empty space

Sheath : pink or greyish sheath, hardly stains with Giemsa or even not at all

Characteristics :
Small, separated nuclei;
first and last ones clearly oval-shaped

Associated biological signs :

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp.*, Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- The microfilaria usually circulate at night in the periferal bloodstream (bloodsample best taken between 23 and 04 o'clock). Sometimes sub-periodically.
- For patients mostly working at night, the blood is best taken during the daytime.
- Sometimes the microfilaria of *Wuchereria bancrofti* can be found in urine (chyluria).
- In cases of elephantiasis the microfilaria usually cannot be found in the periferal bloodstream anymore!!!
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.
- The microfilaria appear in the bloodstream about 8 to 12 months after initial infestation (maturation period). The adult's lifespan is estimated at more than 25 years. The microfilaria can survive up to 70 days.

Brugia malayi

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- South- and East-Asia
- India

Common name : Malayan filaria

Disease :

- NL : Brugia filariosis
- FR : filariose Brugia
- EN : Malayan filariasis
- ES : filariasis de Malaia

Final host :

- Man
- Certain monkeys
- (Cats?)

Intermediate host:

(Vector)

Different kinds of mosquitoes: Family of *Culicidae* (*Mansonia* spp., *Anopheles* spp.)

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm :

Lymphatic system.

Localisation of the microfilaria :

Blood.

Diagnostic possibilities :

- Examination of blood: Direct examination (not very efficient; no identification possible!)
  Thick blood smear: - Giemsa staining
  - Carazzi staining is better, but more complex
  Woo-technique: good concentration-technique (no identification possible!)
  KNOTT-method: very efficient concentration-method.
  After filtration onto a membrane, followed by staining (epidemiological purposes).
- Serology (searching for Ab (mostly IgG4-type) in serum)
- Detection of Ag in blood
- Detection of specific DNA, using molecular techniques (PCR)
- Detection of adult worms and abnormal lymphatic dilatations, using medical imaging

Morphology of the microfilaria (Giemsa):

Dimensions : 175-230 µm x 5-6 µm

Tail : stretched, with two strongly isolated terminal nuclei

Head : long empty space (≈ 2x width of the larva)

Sheath : strongly coloured (but can be absent in sub-periodical microfilaria)

Characteristics : usually strongly rolled up in thick blood smear (not in KNOTT)

Associated biological signs :

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp., *Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- The microfilaria usually circulate at night in the periferal bloodstream (bloodsample best taken between 23 and 04 o'clock). *Brugia malayi* is sub-periodic in Indonesia, Maleisia, the Filippines and Thaïland (the microfilaria can always be found in the periferal bloodstream, but especially at night).
- In 50% of the sub-periodical microfilaria of *B. malayi* the sheath is absent. In the periodical microfilaria of *B. malayi*, the sheath usually is present, but prevents correct staining of the nuclei.
- For patients mostly working at night, the blood is best taken during the daytime.
- Sometimes the microfilaria of *Brugia malayi* can be found in urine.
- In cases of elephantiasis the microfilaria usually cannot be found in the periferal bloodstream anymore!!!
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.
- It takes 67 to 98 days for the larvae to become adult worms. The adult's lifespan is estimated at more than 15 years.

Brugia timori

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- Indonesian archipel: from Timor to Bali, including the satellite-islands

Common name :

Timorean filaria

Disease :

- NL : Timor filariosis
- FR : filariose du Timor
- EN : Timorean filariasis
- ES : filariasis de Timor

Final host :

- Man
- ?

Intermediate host:

(Vector)

Mosquitoes of the family of *Culicidae* (*Anopheles* spp.)

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm :

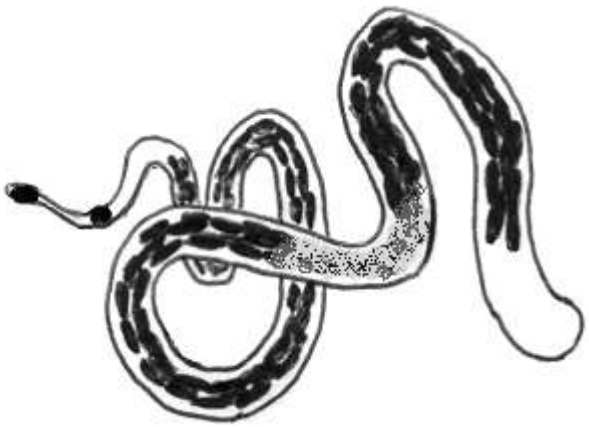
Lymphatic system.

Localisation of the microfilaria :

Blood.

Diagnostic possibilities :

- Examination of blood: Direct examination (not very efficient; no identification possible!)
  Thick blood smear: - Giemsa staining is a good technique, but the sheath usually doesn't take up the colour.
  - Carazzi staining is better, but more complex.
  Woo-technique: good concentration-technique (no identification possible!)
  KNOTT-method: very efficient concentration-method.
  After filtration onto a membrane, followed by staining (epidemiological purposes).
- Serology (searching for Ab (especially IgG4-type) in serum)
- Detection of Ag in blood
- Detection of specific DNA, using molecular techniques (PCR)
- Detection of adult worms and abnormal lymphatic dilatations, using medical imaging



Morphology of the microfilaria (Giemsa):

Dimensions : 265-325 µm x 4,4-6,8 µm

Tail : stretched, with two strongly isolated terminal nuclei kernen

Head : long empty space (≈ 2x to 3x width of the larva)

Sheath : present, but hardly stains with Giemsa or even not at all

Characteristics :
usually strongly rolled up in thick blood smear (not in KNOTT); nuclei stain poorly at ⅓ of the microfilaria's length

Associated biological signs :

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp., *Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- The microfilaria of *Brugia timori* circulate strictly at night in the periferal bloodstream (bloodsample best taken between 23 and 04 o'clock). For patients mostly working at night, the blood is best taken during the daytime.
- Sometimes the microfilaria of *Brugia timori* can be found in urine.
- In cases of elephantiasis the microfilaria usually cannot be found in the periferal bloodstream anymore!!!
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.

Mansonella streptocerca

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- Rainforests of West- and Central Africa

Common name : (Filaria)

Disease : No specific name (non or only slightly pathogenic)

Final host :

- Man
- Some monkey species

Intermediate host:

(Vector)

Mosquitoes (*Culicoides* spp.)

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm : Subcutaneous tissues.

Localisation of the microfilaria : Skin.

Diagnostic possibilities :

- Identification of the microfilaria in skin-biopsies (skin-snip).
- Identification of the microfilaria in deep scarifications.

Morphology of the microfilaria (Giemsa):

Dimensions : 180-240 µm x 5-6 µm

Tail : blunt, usually curled with about 8 nuclei in line

Head : filled with nuclei

Sheath : absent

Characteristics : microfilaria is crosier-shaped

Associated biological signs :

(non or only slightly pathogenic)

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp., *Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- *Dipetalonema* is an ancient name for the group containing (amongst others) *Mansonella* spp. (infesting man) and *Microfilaria* spp. (infesting animals).
- No specific name exists for this disease, as *M. streptocerca* is considered non or only slightly pathogenic. It should however be differentiated from pathogenic microfilaria.
- When both *Onchocerca volvulus* and *Mansonella streptocerca* infest a person, these two microfilaria are not always found in the same skin-regions.
- *Mansonella streptocerca* is an aperiodic microfilaria.
- *Mansonella streptocerca* is often found together with other microfilaria.
- Deep scarifications always contain a small amount of blood, which explains the possibility of finding blood-microfilaria in such a preparation.
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.

Mansonella ozzardi

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- Small regions in Central- and South-America
- Islands of the Pacific and the Caribees

Common name : (Filaria)

Disease : No specific name (non or only slightly pathogenic)

Final host :

- Man
- Some monkey species

Intermediate host:

(Vector)

Mosquitoes (*Culicoides* spp. and *Simulium* spp.)

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm : Abdominal cavity.

Localisation of the microfilaria : Blood.

Diagnostic possibilities :

- Identification of the microfilaria in blood:
  - Direct examination (not very efficient; no identification possible!)
  - Thick blood smear: - Giemsa staining.
    - Carazzi staining is better, but more complex.
  - Woo-technique: good concentration-technique (no identification possible!)
  - KNOTT-method: very efficient concentration-method.
  - After filtration onto a membrane, followed by staining (epidemiological purposes).

Morphology of the microfilaria (Giemsa):

Dimensions : 160-205 µm x 3-5 µm

Tail : empty and pointed tail (not always quite clear)

Head : filled with nuclei

Sheath : absent

Characteristics : nuclei more or less mosaic-shaped

Associated biological signs :

(non or only slightly pathogenic)

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp., *Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- *Dipetalonema* is an ancient name for the group containing (among others) *Mansonella* spp. (infesting man) and *Microfilaria* spp. (infesting animals).
- No specific name exists for this disease, as *M. ozzardi* is considered non or only slightly pathogenic. It should however be differentiated from pathogenic microfilaria.
- *Mansonella ozzardi* is an aperiodic microfilaria.
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.

Mansonella perstans

SuperFamily : Filarioidea

Class : Nematodes

Geographic distribution :

- Tropical and Northern-Africa
- Central- and South-America

Common name : (Filaria)

Disease :

No specific name
(non or only slightly pathogenic)

Final host :

- Man
- Certain monkeys

Intermediate host:

(Vector)

Mosquitoes
(*Culicoides* spp.)

Transmission :

Active penetration of the skin (or via the sting-wound) by infectious larvae, placed on the skin during the blood-meal of an infested vector.

Localisation of the adult worm :

Abdominal cavity, pleural cavity, (pericard)

Localisation of the microfilaria :

Blood.

Diagnostic possibilities :

- Identification of the microfilaria in blood:
  Direct examination (not very efficient; no identification possible!)
  Thick blood smear: - Giemsa staining.
  - Carazzi staining is better, but more complex.
  Woo-technique: good concentration-technique (no identification possible!)
  KNOTT-method: very efficient concentration-method.
  After filtration onto a membrane, followed by staining (epidemiological purposes).

Morphology of the microfilaria (Giemsa):

Dimensions : 150-200 µm x 3-5 µm

Tail : blunt tail, last nucleus seems somewhat bigger, more intensely coloured (maximum 4 nuclei in line)

Head : filled with nuclei

Sheath : absent

Characteristics :
nuclei are mosaic-shaped

Associated biological signs :

(non or only slightly pathogenic)

- Persisting hypereosinophilia during infestation

Possible confusion with :

- Other microfilaria
- Zoonoses (*Dirofilaria* spp.*, Microfilaria* spp.)
- Thread-like artefacts (cotton-threads etc.)
- Fungi (*Helicosporium*)
- Helminth-larvae during their migration-phase

Remarks :

- *Dipetalonema* is an ancient name for the group containing (among others) *Mansonella* spp. (infesting man) and *Microfilaria* spp. (infesting animals).
- No specific name exists for this disease, as *M. perstans* is considered non or only slightly pathogenic. It should however be differentiated from pathogenic microfilaria.
- *M. perstans* is often found together with *Loa Loa* (or other microfilaria).
- *Mansonella perstans* is a nocturnally subperiodic microfilaria.
- When a thick bloodsmear is prepared with blood containing **NO** anticoagulants, the microfilaria of *M. perstans* often are strongly rolled up. When using blood **WITH** anticoagulants, the microfilaria of *M. perstans* usually are nicely stretched out.
- Serological tests do not discriminate between the different species of microfilaria. Also cross-reactivity with other nematodes and with *E. granulosus* exists.
- It takes several months for the larvae to become adult worms.

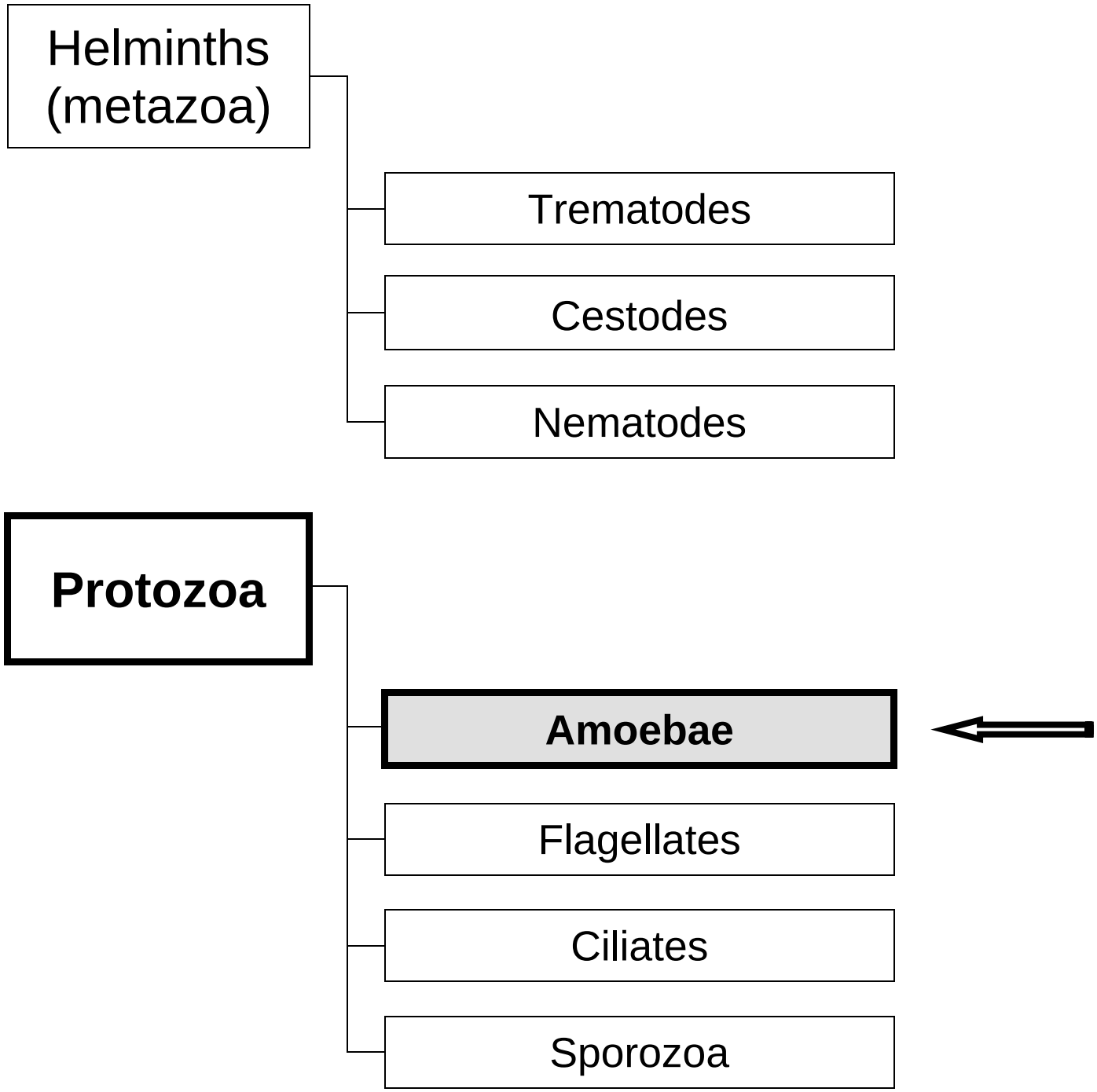
Characteristics of common human microfilaria species

Species Characteristic	***Wuchereria bancrofti***	***Brugia malayi***	***Brugia timori***	***Loa loa***	***Mansonella ozzardi***	***Mansonella perstans***	***Mansonella streptocerca***	***Onchocerca volvulus***
Geographical distribution	Tropical and Subtropical countries (except desert) Turkey (rare ?)	South and East, Indian subcontinent	Indonesian archipel from Timor to Bali and satellite-islands	Central and West Africa (rain forests)	Central and South America, Islands of the Pacific and Caribees	Central and West Africa, Central and South America	Central and West Africa (rain forests)	Central and West Africa, Central and South America
Vectors	Mosquitoes (family of culicidae)	Mosquitoes (family of culicidae)	Mosquitoes (family of culicidae)	Tabanid flies (*Chrysops* spp.)	Biting midges (*Culicoides* spp.) and blackflies (*Simulium* spp.)	Biting midges (*Culicoides* spp.)	Biting midges (*Culicoides* spp.)	Blackflies (*Simulium* spp.)
Habitat (adults)	Lymphatic system	Lymphatic system	Lymphatic system	Subcutaneous tissues, orbit	Subcutaneous tissues	Mesentery	Dermis	Subcutaneous tissues
Habitat (microfilariae)	Blood	Blood	Blood	Blood	Blood	Blood	**Skin**	**Skin**
Diagnostic	Blood examination	Blood examination	Blood examination	Blood examination	Blood examination	Blood examination	Deep scarification or skin biopsy	Deep scarification or skin biopsy
Periodicity of Microfilariae [1]	Nocturnal [2]	Nocturnal [3]	Nocturnal	Diurnal	Aperiodic	Nocturnally subperiodic	Aperiodic	Aperiodic
Sheath	Present	Present	Present	Present	**Absent**	**Absent**	**Absent**	**Absent**
Width (µm)	7.5 -10	5 - 6	4.4 - 6.8	5 - 7	3 - 5	3 - 5	5 - 6	5 - 9
Length (µm)								
Smears	260 [240-300]	220 [175-230]	290 [265-325]	240 [230-250]	180 [160-205]	195 [150-200]	-	-
Formalin 2%	300 [275-320]	270 [240-300]	360 [330-385]	280 [270-300]	225 [200-255]	200 [180-225]	-	-
Biopsy	-	-	-	-	-	-	210 [180-240]	310 [280-320]
Tail	Tapered, anucleate	Tapered, subterminal and terminal nuclei widely separated	Tapered, subterminal and terminal nuclei widely separated	Tapered, Nuclei to end	Pointed, anucleate	Bluntly rounded, nuclei to end	Bluntly rounded, nuclei to end **crosier-shaped**	Pointed, anucleate
Cephalic space	Short	Long (twice as long as broad)	Long (twice [3] as long as broad)	Short	Very short	Very short	Very short	Long
Key features	Nuclei are well distinct, first and last ones oval shaped, Body in smooth curves, sheath unstained in Giemsa	Terminal and subterminal nuclei, sheath **stains** pink in Giemsa	Terminal and subterminal nuclei, sheath unstained in Giemsa	Two hernia, single row of nuclei to end of tail sheath unstained in Giemsa	Anucleated tail	Blunt tail filled with maximum 4 nuclei, mosaic shaped nuclei, last nucleus bigger or more intensely coloured	Blunt tail filled with nuclei (8 nuclei in line)	Empty tail

[1] The microfilariae of some species appear in the blood with a market nocturnal or diurnal periodicity. For nocturnal periodicity, a "night blood specimen" should be examined (maximum between 11 pm and 4 am). For diurnal periodicity, a "day blood specimen" should be examined (maximum between 11 am and 4 pm). Some microfilariae are present all the time in blood, but with an increase during the night (nocturnally subperiodic) or during the day (diurnally subperiodic).

[2] Nocturnally subperiodic in Thailand, diurnally subperiodic in New Caledonia and Polynesia.

[3] Nocturnally subperiodic in some parts of Indonesia, Malaysia, the Philippines and Thailand.


Helminths (metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Entamoeba histolytica

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Worldwide

Common name :

Disease :

- NL : amoebiase
- FR : amibiase
- EN : amoebiasis
- ES : amoebiasis

Final host :

- Man
- Primates
- (Dogs, cats, ...)

Intermediate host:

Without intermediate host and without vector

Transmission :

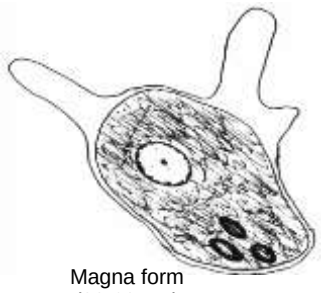
Faeco-oral, mature cysts ingestion.
(Sexual contact, cysts and trophozoites).

Localisation of the parasite :

Minuta form: Intestinal lumen, colon
Magna form: Intestinal mucosa and sub-mucosa (or liver, brain, lungs, …).

Diagnostic possibilities :

- Trophozoites detection in faeces (or in abscess pus, rectal biopsy or sputum) : direct examination.
- Cysts detection in faeces : direct examination, iodine staining, concentration by sedimentation.
- Antigen detection in stool and species differentiation between *E. histolytica* and *E. dispar* in (ELISA, galactose adhesins).
- Detection of specific DNA, using PCR (various samples).
- Serology : antibody detection in serum (IFAT, ELISA, IHA, direct agglutination).
- Differentiation between *E. histolytica* and *E. dispar* (various samples : PCR or ELISA galactose adhesins) .



Magna form
(20-60 µm)

Minuta form
(10-60 µm)

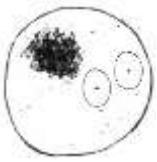
Morphology of the trophozoites :

Size : 10-60 µm (>20 for invasive forms)

Nucleus : 1 not so visible, central karyosome, fine granules of peripheral chromatin, evenly distributed.

Motility : quick, in one direction, finger-like pseudopods

Characteristics :
Ingested RBC for the invasive form.



Morphology of the cysts :

Size : 10-20 µm

Morphology : Spherical or oval

Nucleus : up to 4, usually 1 or 2, central fine karyosome.

Characteristics : Immature cyst with 1 nucleus; sometimes chromatoid body, elongated with bluntly rounded ends; usually diffuse glycogen mass.

Associated biological signs :

- Leukocytosis For extra-intestinal amoebiasis: Leukocytosis, neutrophilia, ESR raised.

Possible confusion with :

- *Entamoeba dispar* (cysts and trophozoites)
- Others cysts of protozoa.
- Others trophozoites of amoebae.
- Leucocytes (cysts)
- Motile macrophages (trophozoites).

Remarks :

- Differentiation between the never pathogenic *E. dispar* and the sometimes pathogenic *E. histolytica*, based on morphologic characteristics of the cysts and trophozoïtes is not possible. Except in case of finding motile amoebae containing RBC which give the diagnosis of invasive amibiasis. Otherwise, differentiation must be based on isoenzymatic or molecular analysis.
- Human infection with *E. dispar* is more widely spread than with *E. histolytica* (about 95% of the infections with *E. histolytica/dispar* found at the Central Laboratory of Clinical Biology of the Institute of Tropical Medicine in Antwerp turn out to be due to *E. dispar*).
- Haematophageous trophozoites are found only in bloody diarrhoeic specimens. Such specimens must be examined without delay, otherwise identification of the trophozoïtes becomes impossible because they lose their motility. Haematophageous trophozoïtes are also found in pus (viscous, yellow, brown or chocolate). Metastatic abscesses (liver, lung, brain,…) contain necrotic tissue, but few leucocytes and trophozoites. These trophozoites are most easily found at the periphery of the abscess. As the abscess is most oftenly punctured in its centre, the microscopic examination of the pus may fail to detect the trophozoites.
- *E. histolytica* (minuta form) remain confined to the intestinal lumen. In some cases, the trophozoïtes invade the intestinal mucosa, or through the bloodstream extra intestinal sites (magna form). Only *E. histolytica* (minuta form) and *E dispar* will produce cysts. Cysts are possibly not present in stool during an intestinal invasive amoebiasis.
- Serology is only useful for invasive amoebiasis (sensitivity of approximately 95% for extra-intestinal invasive infections, 70% for intestinal invasive infections and 10% for asymptomatic persons who are passing *E. histolytica* cysts; specificity of approximately 95%). Detectable *E. histolytica* specific antibodies may persist for months after successful treatment.
- Antigen detection (galactose adhesine) may be useful to distinguish between *E. histolytica* and *E dispar* (sensitivity is about 70% and specificity about 97%)
- Cysts and trophozoites of *Entamoeba.moshkowskii* (a non pathogenic amoeba) and of *E. polecki* (an amoeba of mostly pigs and monkeys) are identical to those of *E. dispar* and to cysts and minuta forms of *E. histolytica*.
- *Entamoeba histolytica* cysts are resistant to the concentration of chlorine commonly used in the purification of domestic water supplies. Filtration of drinking water is an effective method to eliminate these cysts.

Entamoeba dispar

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Primates
- Other animals

Intermediate host:

Without intermediate host and without vector

Transmission :

Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen, colon

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.
- Cysts identification in faeces : direct examination, iodine staining, concentration by sedimentation.
- Differentiation between *E. histolytica* and *E. dispar* : PCR or ELISA (galactose adhesins) .

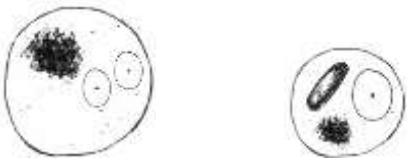
Morphology of the trophozoites :

Size : 10-20 µm.

Nucleus : 1 not so visible, central karyosome, fine granules of peripheral chromatin, evenly distributed.

Motility : quick, in one direction, finger-like pseudopods

Characteristics : Never contain ingested RBC



Morphology of the cysts :

Size : 10-20 µm

Morphology : spherical or oval

Nucleus : up to 4, usually 1 or 2, central fine karyosome.

Characteristics : Immature cyst with 1 nucleus; sometimes chromatoid body, elongated with bluntly rounded ends; usually diffuse glycogen mass.

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- *Entamoeba histolytica* (cysts and trophozoites)
- Others cysts of protozoa.
- Others trophozoites of amoebae.
- Motile macrophages (trophozoites)..

Remarks :

- Differentiation between the never pathogenic *E. dispar* and the sometimes pathogenic *E. histolytica*, based on morphologic characteristics of the cysts and trophozoïtes is not possible. Differentiation must be based on isoenzymatic or molecular analysis.
- Human infection with *E. dispar* is more widely spread than with *E. histolytica* (about 95% of the infections with *E. histolytica/dispar* found at the Central Laboratory of Clinical Biology of the Institute of Tropical Medicine in Antwerp turn out to be due to *E. dispar*).
- Cysts are found in more formed specimens, trophozoïtes are found mainly in diarrhoeic specimens.
- Specimens must be examined without delay, otherwise identification of the trophozoïtes becomes impossible because they lose their motility.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.
- Antigen detection (galactose adhesine) may be useful to distinguish between *E. histolytica* and *E dispar* (sensitivity is around 70% and specificity is around 97%).
- Cysts and trophozoites of *Entamoeba.moshkowskii* (a non pathogenic amoeba) and of *E. polecki* (an amoeba of mostly pigs and monkeys, non pathogenic in humans) are identical to those of *E. dispar* and to cysts and minuta forms of *E. histolytica*.

Entamoeba hartmanni

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man

Intermediate host:

Without intermediate host and without vector

Transmission :

Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.
- Cysts identification in faeces : direct examination, iodine staining, concentration by sedimentation.

Morphology of the trophozoites :

Size : 5-12 µm.

Nucleus : 1 not so visible, central karyosome, fine granules of peripheral chromatin, evenly distributed.

Motility : quick, in one direction, finger-like pseudopods

Characteristics : Never contain ingested RBC

Morphology of the cysts :

Size : 5-10 µm

Morphology : Spherical or oval

Nucleus : up to 4, usually 1 or 2, central fine karyosome.

Characteristics : Immature cyst with 1 nucleus; sometimes chromatoid body, elongated with bluntly rounded ends; usually diffuse glycogen mass.

"Small *histolytica*"

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- *Entamoeba histolytica/dispar* (cysts and trophozoites)
- Others cysts of protozoa.
- Others trophozoites of amoebae.
- Motile macrophages (trophozoites)..

Remarks :

- As non pathogenic parasites, *E. hartmanni* should not be looked for but should be distinguished from the (sometimes) pathogenic *E. histolytica*.
- Accurate measurement of cysts is essential for the correct distinction between *E. histolytica/dispar* and *E. hartmanni*.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.

Entamoeba coli

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Primates
- Dogs
- Pigs

Intermediate host:

Without intermediate host and without vector

Transmission :

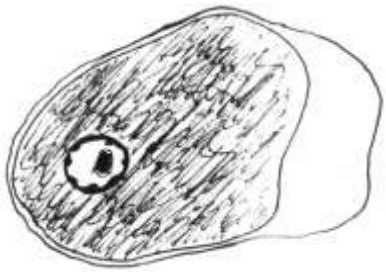
Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.
- Cysts identification in faeces : direct examination, iodine staining, concentration by sedimentation.



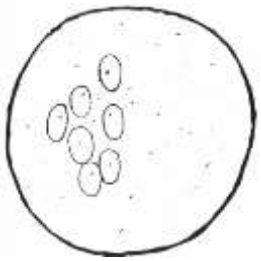
Morphology of the trophozoites :

Size : 15-50 µm.

Nucleus : 1, most of the time large and excentric karyosome, irregular granules of peripheral chromatin.

Motility : Barely motile, large pseudopods

Characteristics : Never contain ingested RBC



Morphology of the cysts :

Size : 10-35 µm.

Morphology : Spherical or oval.

Nucleus : 1 to 8, well visible (out of the ordinary 8 to 16).

Characteristics : Tick shell; sometimes chromatoid body, with sharp or jagged ends; usually diffuse glycogen.

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- Others cysts of protozoa.
- Others trophozoites of amoebae.
- Motile macrophages (trophozoites).

Remarks :

- As non pathogenic parasites, *E. coli* should not be looked for but should be distinguished from the (sometimes) pathogenic *E. histolytica*.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.

Endolimax nanus

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Primates
- Dogs
- Pigs

Intermediate host:

Without intermediate host and without vector

Transmission :

Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.
- Cysts identification in faeces : direct examination, iodine staining, concentration by sedimentation.

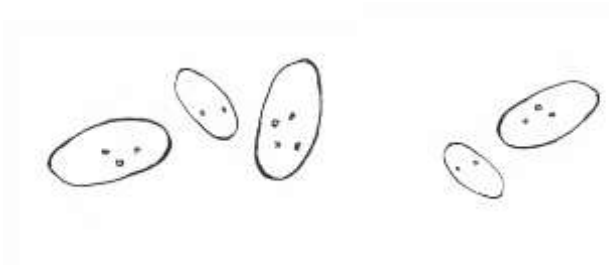
Morphology of the trophozoites :

Size : 6-12 µm.

Nucleus : 1, large and compact karyosome, no peripheral chromatin.

Motility : Slow, only 1 pseudopods.

Characteristics : Never contain ingested RBC



Morphology of the cysts :

Size : 5-10 µm.

Morphology : Oval, sometimes spherical.

Nucleus : 1 to 4, not well visible, (refringent dots).

Characteristics :

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- Others cysts of protozoa.
- Others trophozoites of amoebae.
- Motile macrophages (trophozoites).

Remarks :

- As non pathogenic parasites, *Endolimax nanus* should not be looked for but should be distinguished from the (sometimes) pathogenic *E. histolytica*.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.
- *Endolimax nana*, is the old name for *Endolimax nanus.*

Iodamoeba butschlii

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Primates
- Pigs

Intermediate host:

Without intermediate host and without vector

Transmission :

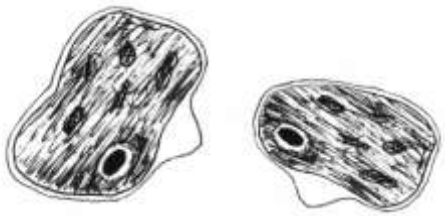
Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.
- Cysts identification in faeces : direct examination, iodine staining, concentration by sedimentation.



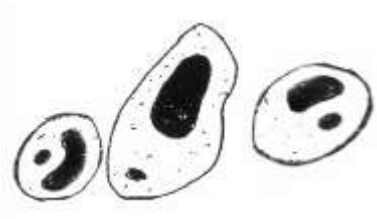
Morphology of the trophozoites :

Size : 8-20 µm.

Nucleus : One not so visible, large karyosome, no peripheral chromatin.

Motility : Slow, transparent pseudopods.

Characteristics : Never contain ingested RBC



Morphology of the cysts :

Size : 5-20 µm.

Morphology : Variable, oval, spherical.

Nucleus : One, compact.

Characteristics : Large glycogen vacuole (stained reddish-brown by iodine solution)

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- Others cysts of protozoa.
- Others trophozoites of amoebae.
- Motile macrophages (trophozoites).

Remarks :

- As non pathogenic parasites, *Iodamoeba butschlii* should not be looked for but should be distinguished from the (sometimes) pathogenic *E. histolytica*.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.
- *Pseudolimax butschlii*, is the old name for *Iodamoeba butschlii.*

Entamoeba gingivalis

Family : Entamoebidae

Class : Rhizopodea

Geographic distribution :

Cosmopolitic

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Dog
- Horse

Intermediate host:

Without intermediate host and without vector

Transmission :

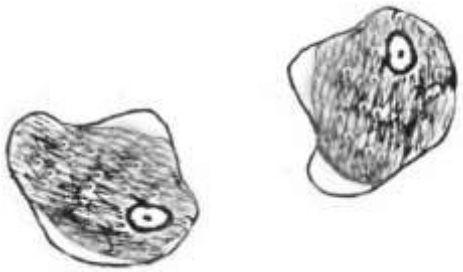
Direct oral transmission (kissing).

Localisation of the parasite :

Mouth cavity.

Diagnostic possibilities :

- Identification of trophozoïtes in saliva, using direct examination
- Identification of trophozoïtes in tooth-scrapings, using direct examination



Mophology of the trophozoïtes :

Dimensions : 10-25 µm

Nucleus : small karyosoma, surrounded by fine peripheral chromatin

Motion : directed movement, most of the time using different pseudopoda at the same time

Morphology of the cysts :

Associated biological signs :

Non pathogenic

Possible confusion with :

- *Entamoeba histolytica* (pulmonary amoebiasis)

Remarks :

- The presence of *Entamoeba gingivalis* usually indicates poor mouth hygiene.
- ATTENTION: *Entamoeba gingivalis* is to be differentiated from *Entamoeba histolytica* coming from the lungs (pulmonary amoebiasis), the latter containing RBCs!!!

Naegleria fowleri

Family : Vahlkampfiidae

Class : Rhizopodea

Geographic distribution :

Cosmopolitic

Common name :

Disease :

Exceptional !

- NL : primaire amoeben-meningo-encefalitis
- FR : méningo-encéphalite amibienne primitive
- EN : primary amebic meningoencephalitis
- ES : meningoencefalitis amibiana primaria

Final host :

Freeliving organism

Accidentally : Man

Intermediate host :

Without intermediate host and without vector

Transmission :

Uptake of infested water through the nose.

Localisation of the amoeboïd trophozoïte:

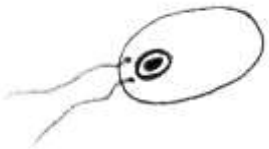
Central Nervous System.

Diagnostic possibilities :

- Detection of the amoeboïd trophozoïtes in CSF using direct examination, after Giemsa-staining, using cultures.
- Identification of the amoeba using immunofluorescence (marked specific Ab).

Mophology of the amoeboïed trophozoïtes : in CSF

Dimensions :	10 – 20 µm.
Movement :	Very actif ; rounded «exploding» pseudopodes
Nucleus :	large with a big karyosoma
Characteristics :	multiple vacuoles in the cytoplasm



Mophology of the flagellated trophozoïtes : in water or more exceptionally in CSF

Dimensions :	10 – 20 µm.
Nucleus :	1 nucleus.
Characteristics:	2 to 4 flagella

Mophology of the cysts : in water

Dimensions :	10 – 20 µm.
Shape :	Oval
Shell :	Double, thick and smooth shell
Nucleus :	1 single nucleus

Associated biological signs:

- purulent CSF
- hyperalbuminorachia
- normal or lowered glycorachia

Possible confusion with :

- *Acanthamoeba* spp.
- *Balamuthia mandrillaris*
- Motile macrofages
- Cysts and trophozoïtes of other amoeba en flagellates (contaminants)

Remarks :

- *Naegleria* spp. and amoeba of *Acanthamoeba* spp. are free-living organisms, usually found in water (lakes, swimming-pools, water-distribution, water from heating installations and aircoes,…). Their optimal multiplying temperature is ±35°C. *Naegleria fowleri,* the only *Naegleria* sp. that can occasionally be pathogenic for man, is found only in warm water (>20°C), A*canthamoeba* species can also be found in colder water. *Balamuthia mandrillaris* was never isolated from the external environment up till 2003.
- Primary amoebic-meningo-encefalitis is usually encountered as small epidemics (persons that came simultanuously in contact with infested water (swimming-pools,…)).
- *Naegleria fowleri* is a strange parasiet, as it has 3 different stages in its evolutionary cycle: the amoeboïd trophozoïte (parasitic form), the flagellated trophozoïte (free-living and multiplying form, exceptionally also found in CSF) and the cyst (protected form in the external environment). The cysts are well-protected against drought, but are sensitive to chlorine (4 ppm).
- Primary amoebic-meningo-encefalitis causes purulent CSF, containing especially poly-nuclear WBCs (500-20.000 cells/mm³), hyperalbuminorachia, normal or lowered glycorachia, a negative bacteriological examination of CSF with presence of numerous motile amoeba.

Acanthamoeba spp.

Family : Hartmanellidea

Class : Rhizopodea

Geographic distribution :

Cosmopolitic

Common name :

Disease:
Very exceptional encephalitis !

- NL : amoeben keratitis
  [granuleuze amoeben-encefalitis]
- FR : ulcère cornéen amibien
  [encéphalite granuleuse amibienne]
- EN : keratitis and corneal amebic ulcer
  [chronic granulomatous amebic encephalitis]
- ES : ulceras corneales amibiana
  [encefalitis amibiana granulosa]

Final host :

Freeliving organism

Accidentally : Man

Intermediate host :

Without intermediate host and without vector

Transmission :

Direct eye contact

Localisation of the amoeboïd trophozoïte and cyst:

Skin, eye [,central nervous system].

Diagnostic possibilities :

- Detection of amoeboïd trophozoïtes and cysts in cornea-scrapings:
  - direct examination, after Giemsa-staining,…
- Detection of amoeboïd trophozoïtes and cysts in biopsies (skin, cornea, brain-tissue) :
  - direct examination, after Giemsa-staining,…
- Identification of the amoeba using immunofluorescence (marked specific Ab).
- [Culture].
- [Detection of amoeboïd trophozoïtes and cysts in CSF:
  - direct examination, after Giemsa-staining,…]

Mophology of the amoeboïd trophozoïtes :

Dimensions :	10 – 40 µm.
Movement:	slow, granular pseudopodes
Nucleus :	large with a big karyosoma
Characteristics:	efilated or filamentous pseudopodes; multiple vacuoles

Mophology of the cysts :

Dimensions :	10 – 20 µm.
Form :	oval
Shell :	double shell with polygonal outer structure
Nucleus :	1 single nucleus, usually hardly visible.
Characteristics:	polygonal aspect

Associated biological signs :

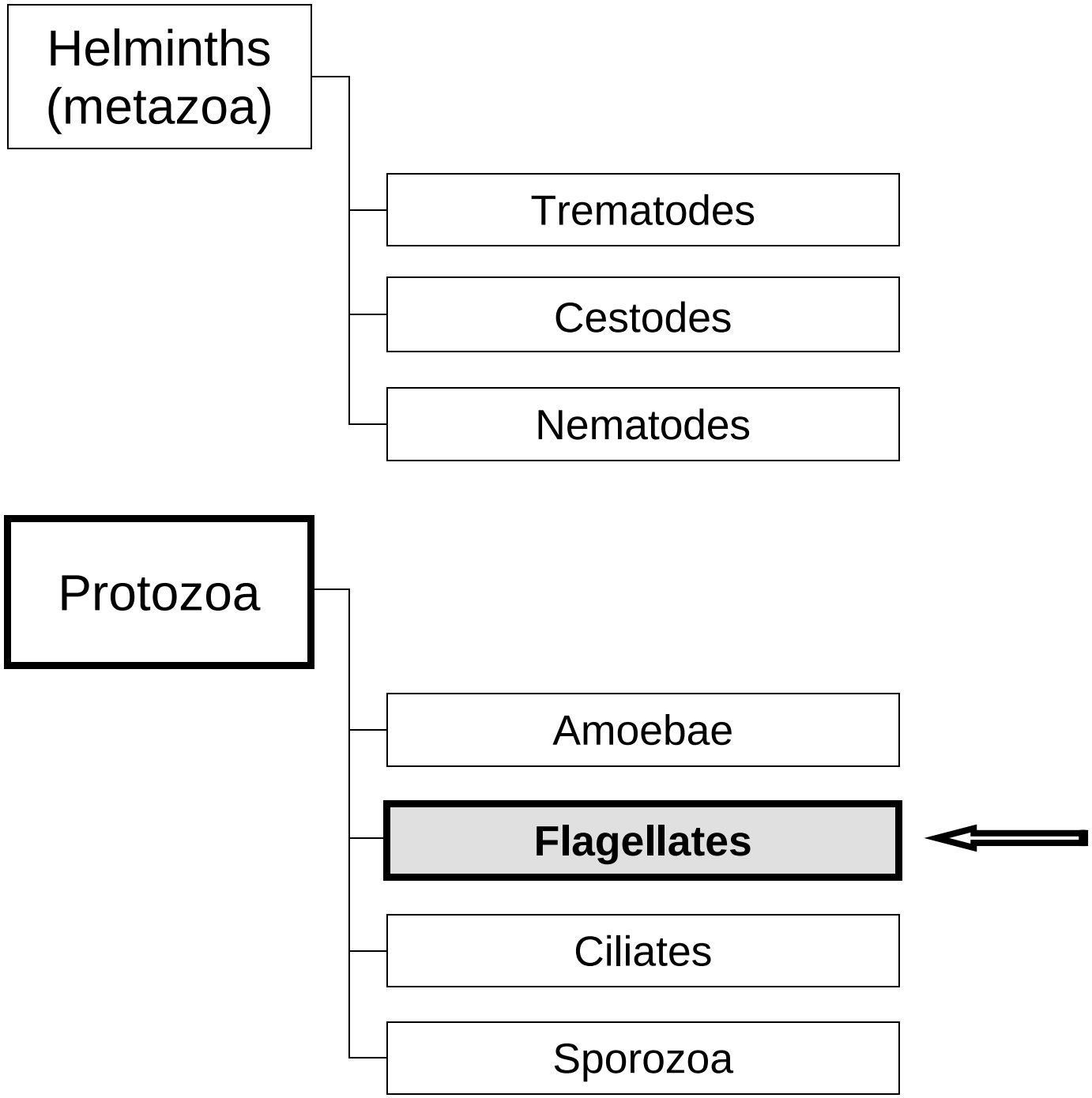
- hyperalbuminorachia
- normal or lowered glycorachia

Possible confusion with :

- *Naegleria* spp.
- *Balamuthia mandrillaris*
- plantspores
- Motile macrofages
- Cysts and trophozoïtes of other amoeba (contaminants)

Remarks :

- *Naegleria* spp. and amoeba of *Acanthamoeba* spp. are free-living organisms, usually found in water (lakes, swimming-pools, water-distribution, water from heating installations and aircoes,…). Their optimal multiplying temperature is ±35°C. *Naegleria fowleri,* the only *Naegleria* sp. that can occasionally be pathogenic for man, is found only in warm water (>20°C), A*canthamoeba* species can also be found in colder water. *Balamuthia mandrillaris* was never isolated from the external environment up till 2003.
- Several *Acanthamoeba* spp. can evolve pathogenically (opportunistic): *A. polyphaga, A. castellani, A. culbertsoni, A. astronyxis, A. hatchetti, A. rhysodes*,…
- In contrast to *Naegleria fowleri*, *B. mandrillaris* and the amoeba of *Acanthamoeba* spp. have only two evolutionary stages: an amoeboïd and a cyst-stage.
- The amoeba of *Acanthamoeba* spp. and of *Balamuthia mandrillaris* are morphologically identical. Both of these amoeba can cause keratitis after damage to the cornea (scratchings, trauma, contactlenses,…) or encephalitis (opportunistic, in case of immuno-deficiëncy).
- Granulary amoebic-encefalitis is found in combination with clear CSF, containing especially mono-nuclear WBCs, hyperalbuminorachia, normal or lowered glycorachia, a negative bacteriological examination of CSF with presence of motile amoeba (hard to find however) and/or polygonal-shaped cysts.


Helminths (metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and bacteria
Unknown classification

Dientamoeba fragilis

Superfamily : Monocercomonadidea

Class : Rhizopodea

Geographic distribution :

Cosmopolitic

Common name :

Disease :

No specific name exists for this (still hypothetical) disease.

Final host :

- Man

Intermediate host:

Without intermediate host and without vector ?

Transmission :

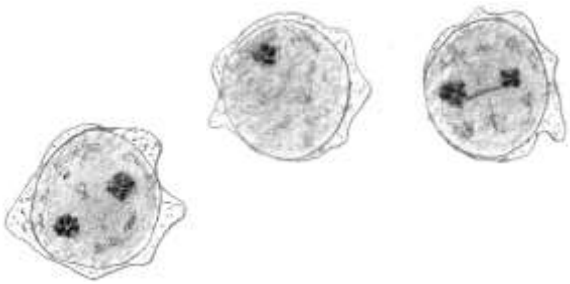
- Faeco-oral transmission of the trophozoïtes?
- The hypothesis that transmission occurs, using helminth eggs (such as those of *E. vermicularis*) is yet to be proven!

Localisation of the trophozoïte :

Lumen of the colon

Diagnostic possibilities :

- Detection of the trophozoïtes in permanently stained slides of faeces (e.g. iron-hematoxylin-staining; see p.104).
- Identification of the trophozoïtes in permanently stained slides after detection in direct wet smears
- in vitro cultivation on xenic media
- Detection of specific DNA in faeces, using PCR
- Serology: detection of specific Ab



Mophology of the trophozoïtes: in permanently stained slides

Dimensions : 4-20 µm

Nucleus : 1 to 2 nuclei, clearly fragmented (4 to 8 fragments), sometimes connected by a centrodesmose; no peripheral chromatin

Movement : very actif amoeboïd movement, using pseudopodes (in direct wet smears); immobile after fixation and/or staining

Characteristics: About 60% of the trophozoïtes has two typically fragmented nuclei

Morfologie van de cysten :

Associated biological signs:

- Pathogenic capacity not yet proven!

Possible confusion with :

- amoebic trophozoïtes
- Motile macrofages (in direct wet smears)
- Cysts and trophozoïtes of *Endolimax nanus* (in permanently stained slides)

Remarks :

- The pathogenic capacity of this flagellate has not yet been proven.
- As *D. fragilis* does not form cysts and as the trophozoïtes are quite fragile, the stoolsample should be fixed **immediately** after production (e.g. in SAF-fixative; see p.124).
- To identify *D. fragilis* the typically fragmented nuclei should be visualized, by using permanently stained slides (e.g. iron-hematoxylin staining; see p.104).
- The staining of faecal specimens is very time-consuming and the reading of the slides demands a high level of experience.
- While having a typical amoeboïd aspect and lacking any flagella, this parasite is classified amongst the flagellates, based on its genetic characteristics

.

Giardia lamblia

Family : Hexamitidae

Class : Zoomastigophorea

Geographic distribution :

Worldwide

Common name :

Disease :

- NL : giardiase / lambliase
- FR : giardiase
- EN : giardiosis
- ES : giardiasis

Final host :

- Man (reservoir)
- Primates
- Pigs
- Rodents (reservoir ?)

Intermediate host :

Without intermediate host and Without vector

Transmission :

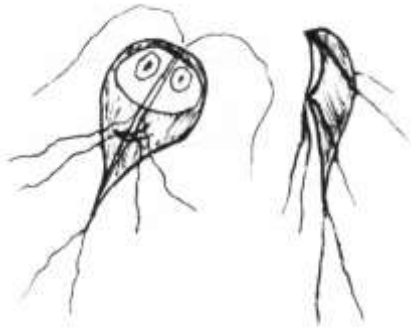
Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Duodenum and first part of the jejunum

Diagnostic possibilities :

- Trophozoites detection in faeces : direct examination.
- Cysts detection in faeces : direct examination, iodine staining, concentration by sedimentation.
- Trophozoites detection in duodenal aspirate (or using a string-test (Enterotest®)): direct examination.
- Antigen detection in faeces (ELISA).
- Serology : antibody detection in serum (IFAT).
- Detection of specific DNA, using PCR.



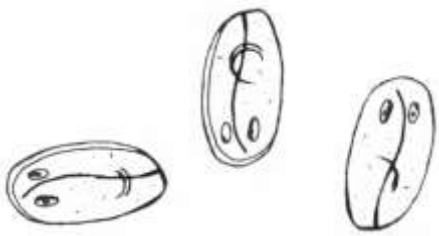
Morphology of the trophozoites :

Size : 10-20 µm x 6-8 µm x 1-4 µm

Nucleus : 2 anteriorly placed

Motility : In a definite direction, as a falling dead leaf

Characteristics : pear-shaped (frontal view), spoon–shaped (lateral view), 8 flagella.



Morphology of the cysts :

Size : 8 -19 µm x 7-10 µm

Morphology : oval

Nucleus : 2 to 4 anteriorly placed

Characteristics : Axostyle in S-shape, median bodies comma-shaped. Cytoplasmic retraction is common (shell appears to be double walled).

Associated biological signs :

- Usually without any symptoms
- Watery diarrhoea without blood or mucus
- Hypovitaminosis, especially in children (malabsoption of fatty acids)

Possible confusion with :

- *Chilomastix mesnili* (trophozoites)
- Other trophozoites of non pathogenic flagellates
- Other cysts of protozoa

Remarks :

- Cysts are found in more formed specimens, trophozoïtes are found mainly in diarrhoeic specimens.
- Fresh specimens must be examined without delay, otherwise identification of the trophozoïtes becomes impossible because they lose their motility.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.
- The cysts are excreted irregularly. The sensitivity for microscopic examination of only one specimen is about 75%. Several specimens collected at different times may need to be examined.
- As *E. histolytica, Giardia* cysts are resistant to the concentration of chlorine commonly used for the purification of domestic water supplies.
- *Giardia intestinalis, Giardia duodenalis* and *Lamblia intestinalis* are old names for *Giardia lamblia.*

Chilomastix mesnili

Family: Retortamonadidea

Class : Retortamonadea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Monkeys
- Pigs

Intermediate host:

Without intermediate host and without vector

Transmission :

Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.
- Cysts identification in faeces : direct examination, iodine staining, concentration by sedimentation.

Morphology of the trophozoites :

Size : 6-24 µm x 4-7 µm

Nucleus : 1 anteriorly placed

Motility : In one definite direction, in a spiral

Characteristics :
Pear-shaped with a sharp, pointed end, 5 flagella : 4 anterior and 1 posterior, the last being attached to the body by an ondulating membrane.

Morphology of the cysts :

Size : 4-10 µm

Morphology : Pear-shaped

Nucleus : One, central

Characteristics :
Cytoplasm with fibrils (atrophic flagella).

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- *Giardia lamblia* (trophozoites).
- Other non pathogenic flagellates (trophozoites)
- Other cysts of protozoa.

Remarks :

- As non pathogenic parasites, *Chilomastix mesnili* should not be looked for but should be distinguished from pathogenic amoebae or flagellates.
- Iodine stain will kill any trophozoïtes immediately, but stains the internal structure of the cysts.
- *Retortamonas hominis* (non pathogenic parasite for man and monkey's) and *Enteromonas hominis* (non pathogenic parasite for man and animals) are similar but smaller (4 -10 µm).

Trichomonas vaginalis

Family : Trichomonadidae

Class : Parabasalea

Geographic distribution :

Worldwide

Common name :

Disease :

- NL : trichomoniase (vaginitis, uretritis)
- FR : trichomoniase
- EN : trichomonosis
- ES : tricomoniasis urogenital

Final host :

- Man

Intermediate host:

Without intermediate host and without vector

Transmission :

Sexual transmission

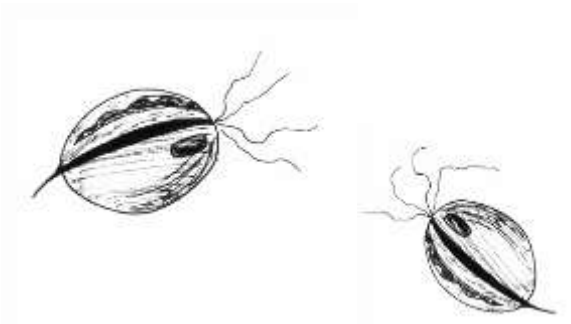
Rare mechanical transmission (wet objects : towels, gynaecological instruments, …)

Localisation of the parasite :

Urogenital tract.

Diagnostic possibilities :

- Trophozoites detection in vaginal discharges or in urethral pus : direct examination.
- Trophozoites detection in urine : Direct examination, concentration by sedimentation.
- [Antigen detection on urine or on vaginal discharge : ELISA, IFAT, …].
- [Culture].
- Detection of specific DNA, using PCR.



Morphology of the trophozoites :

Size : 5-19 µm x 3-12 µm

Nucleus : 1 anteriorly placed, hardly visible.

Motility : Often slight (on the spot) and not progressional.

Characteristics : 4 anteriorly placed flagella, axostyle, ondulating membrane.

NO CYST STAGE

Morphology of the cysts :

NO CYST STAGE

Associated biological signs :

- Purulent vaginal discharge, prurit.
- Usually asymptomatic (men).

Possible confusion with :

- Other *Trichomonas* spp. (contamination).
- Other flagellates (faecal or water contaminants).

Remarks :

- Specimens must be examined without delay, otherwise identification of the trophozoïtes becomes impossible (loss of motility).
- As the parasites live in the urethra, the first drops of urines (after a period of 4 to 5 hours in which the patient did not urinate) have to be examined for trophozoites.

Pentatrichomonas hominis

Family : Trichomonadidae

Class : Parabasalea

Geographic distribution :

Worldwide

Common name :

Disease :

Non pathogenic

Final host :

- Man
- Monkeys
- Pigs
- Dogs
- Rodents
- …

Intermediate host:

Without intermediate host and without vector

Transmission :

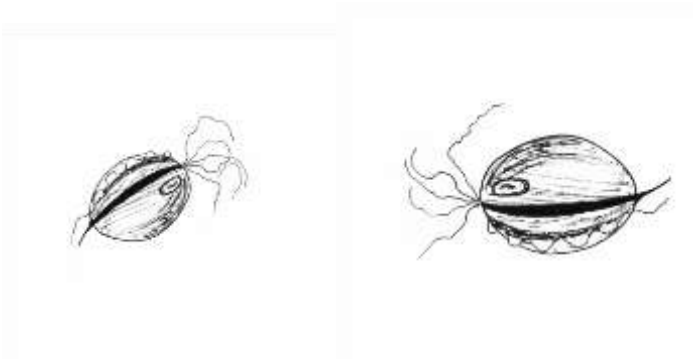
Faeco-oral, **Trophozoites** ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites identification in faeces : direct examination.



Morphology of the trophozoites :

Size : 8-15 µm x 4-6µm

Nucleus : 1 anteriorly placed, hardly visible.

Motility : Often slight (on the spot) and not progressional.

Characteristics : 5 anteriorly placed flagella, 1 posteriorly placed flagellum (ondulating membrane), axostyle

NO CYST STAGE

Morphology of the cysts :

NO CYST STAGE

Associated biological signs :

NON PATHOGENIC

Possible confusion with :

- *Giardia lamblia.*
- Other (non pathogenic) flagellates.

Remarks :

- As non pathogenic parasites, *Pentatrichomonas hominis* should not be looked for but should be distinguished from pathogenic flagellates.
- Pentatrichomonas hominis stays motile quite long in faeces (no cyst stage).
- Another non pathogenic parasite, with a similar morphology (*Trichomonas tenax*) lives in the oral cavity. *Trichomonas elongata* and *Trichomonas buccalis* are old names for this parasite.
- *Trichomonas hominis* and *Trichomonas intestinalis* are old names for *Pentatrichomonas hominis.*

Trypanosoma brucei gambiense

Family : Trypanosomatidae

Class : Mastigophorea

Geographic distribution :

West and Central Sub-Saharian Africa
(between 15°N and 25°S)
Dense vegetation and **riverbanks**

Common name :

West African sleeping sickness

Disease:

- NL : West-Afrikaanse trypanosomiase.
- FR : Trypanosomiase chronique de l'Afrique de l'Ouest.
- EN : West African Trypanosomiasis
- ES : Enfermedad del sueño forma Gambiana.

Final host:

- Man
- (Domestic and semi-domestic animals)
- (Wild animals: buffaloes, etc.)

Intermediate host:

(Vector)

Tsetse fly

(*Glossina palpalis* Group)

Transmission:

- Trypomastigotes injected with saliva through the skin when an infested tsetse fly takes a blood meal.
- Blood transfusion (fresh blood).
- (Congenital transmission: rare).
- (Mechanical transmission by an insect bite: exceptional)

Localisation of the parasite :

- Stage I: lymph and blood.
- Stage II: (lymph and blood), central nervous system

Diagnostic possibilities:

- Serology (detection of Ab in serum or blood): CATT (screening method), latex (especially for Nigeria), ELISA, IF, …
- Examination of lymph node aspirate for trypomastigotes (cervical lymph glands: Winterbottom's sign).
- Examination of blood for trypomastigotes :
  - in a wet smear (low sensitivity).
  - in a (thin or) thick blood film stained with Giemsa.
  - Woo (micro-haematocrite centrifugation).
  - QBC.
  - mini-column (mAECT). For this technique, heparine should be used as an anticoagulant.
- Examination of cerebrospinal fluid for trypomastigotes (diagnostic and stage determination) :
  - in a wet smear (low sensitivity).
  - after single or double centrifugation.
- Detection of specific DNA using PCR

Morphology of the trypomastigotes (Giemsa):

Dimensions : 15-25 µm

Cytoplasm : Pale blue, with a flagellum and an undulating membrane.

Contents : A large central (purple) nucleus and a small kinetoplast (red).

Characteristics : Trypanosomes may show a variety of forms (pleomorphic).

Associated biological signs :

- Stage I: (erythrocyte sedimentation rate ↑ [IgM↑], moderate leukocytosis, thrombocytopenia, anaemia, …)
- Stage II: (idem + CSF perturbation)

Possible confusion with:

- *Trypanosoma brucei rhodesiense*
- Animal's trypanosomiases (*T. brucei brucei*,...)
- Motile bacteria (*Borrelia* spp. or water contaminants) or exflagellation of *Plasmodium* spp. for wet smear.
- *Trypanosoma cruzi* (South America)
- *Trypanosoma rangeli* (South America)

Remarks :

- Microscopically, the trypanosomes of *T. b. gambiense* and *T. b. rhodesiense* look alike. Clinical status and geographical location will give the species diagnosis.
- Because of the risks associated with the drug treatment, particularly in stage II, it is essential to confirm the diagnosis parasitologically (microscopically) before commencing treatment. A number of serologic assays are available to aid in the diagnosis of trypanosomiasis, but their variable sensitivity and specificity mandate that decisions about treatment be based on demonstration of the parasite. The parasitological confirmation by a second technician may be useful.
- Because of the risks associated with the drug treatment, at least 4 of the 5 characteristics must be present for a sure diagnosis in Giemsa stained films: dimension, nucleus, kinetoplast, cytoplasm, flagellum (and undulating membrane).
- Because of the host immunity response, several specimens may need to be examined before detecting the trypanosomes (successive populations with different surface antigens).
- The examination for motile trypanosomes must be done as soon as possible: Trypanosomes are unable to survive for more than 15-20 minutes outside of the host's body (glucose consumption). Trypanosomes are also immobilised by sunlight.
- The stage determination is based on the central nervous system invasion. Following the WHO recommendations for stage determination, the CSF has to be examined at least on white blood cell number, total protein concentration, presence of trypanosomes. Since there is no close relationship between these 3 parameters, all of them should be examined. Other parameters may be useful to use : Measurement of IgM concentration, detection of specific antibodies, …
- *T. b. gambiense* provokes a chronic illness which, without treatment, always ends in the patient's death. The *T. b. gambiense* type II strain (found at the Ivory Coast) however causes an illness with a more acute evolution.

Trypanosoma brucei rhodesiense

Family : Trypanosomatidae

Class : Mastigophorea

Geographic distribution :

East-Africa
(between 15°N and 25°S)
(savanna)

Common name :

East African sleeping sickness

Disease:

- NL : Oost-Afrikaanse trypanosomiase
- FR : trypanosomiase aigue de l'Afrique de l'Est
- EN : East African Trypanosomiases
- ES : enfermedad del sueño forma Rodesiana

Final host:

- Wild animals
- (Domestic animals)
- (Man)

Intermediate host:

(Vector)

Tsetse fly

(*Glossina morsitans* Group)

Transmission:

- Trypomastigotes injected with saliva through the skin when an infested tsetse fly takes a blood meal.
- (Blood transfusion).
- (Mechanical transmission by an insect bite: exceptional).

Localisation of the parasite :

- Stage I: lymph and blood.
- Stage II: (lymph and blood), central nervous system

Diagnostic possibilities:

- Examination of lymph node aspirate for trypomastigotes (less useful for *T. b. rhodesiense*).
- Examination of blood for trypomastigotes :
  - in a wet smear (low sensitivity).
  - in a (thin or) thick blood film stained with Giemsa.
  - Woo (micro-haematocrite centrifugation).
  - QBC.
  - mini-column (mAECT). For this technique, heparine should be used as an anticoagulant.
- Examination of cerebrospinal fluid for trypomastigotes (diagnostic and stage determination) :
  - in a wet smear (low sensitivity).
  - after single or double centrifugation.
- Detection of specific DNA using PCR
- Serology : not very useful because of the fast disease's evolution.

Morphology of the trypomastigotes (Giemsa):

Dimensions : 15-25 µm.

Cytoplasm : Pale blue, with a flagellum and an undulating membrane.

Contents : A large central nucleus and a small kinetoplast (red).

Characteristics : Trypanosomes may show a variety of forms (pleomorphic).

Associated biological signs :

- Stage I: (erythrocyte sedimentation rate ↑ [IgM↑], moderate leukocytosis, thrombocytopenia, anaemia, …)
- Stage II: (idem + CSF perturbation)

Possible confusion with:

- *Trypanosoma brucei gambiense*
- Animal's trypanosomiases (*T. brucei brucei*,...)
- Motile bacteria (*Borrelia* spp. or water contaminants) or exflagellation of *Plasmodium* spp. for wet smear.
- *Trypanosoma cruzi* (South America)
- *Trypanosoma rangeli* (South America)

Remarks :

- Microscopically, the trypanosomes of *T. b. gambiense* and *T. b. rhodesiense* look the same. Clinical status and geographical location will give the species diagnosis.
- Because of the risks associated with the drug treatment, particularly in stage II, it is essential to confirm the diagnosis parasitologically before starting treatment. A number of serological assays are available to aid in the diagnosis of trypanosomiasis, but their variable sensitivity and specificity mandate that decisions about treatment be based on demonstration of the parasite. The parasitological confirmation by a second technician may be useful.
- Because of the risks associated with the drug treatment, at least 4 of the 5 characteristics must be present for a sure diagnosis in Giemsa stained films : dimension, nucleus, kinetoplast, cytoplasm, flagellum and an undulating membrane.
- Because of the host immunity response, several specimens may need to be examined before detecting the trypanosomes (successive populations with different surface antigens).
- The examination for motile trypanosomes must be done as soon as possible: Trypanosomes are unable to survive for more than 15-20 minutes outside of the host body (glucose consumption). Sunlight also immobilizes the trypanosomes.
- The stage determination is based on the central nervous system invasion. Following the WHO recommendations for stage determination, the CSF has to be examined at least on white blood cell number, total protein concentration, presence of trypanosomes. Since there is no close relationship between these 3 parameters, all of them should be examined. Other parameters may be useful : Measurement of IgM concentration, detection of specific antibodies, …
- *T. b. rhodesiense* provokes an acute illness which, without treatment, always ends in the patient's death. The *T. b. rhodesiense* type Zambia strain (found at Zambia and Malawi) however causes an illness with a more chronic evolution.

Trypanosoma cruzi

Family : Trypanosomatidae

Class : Mastigophorea

Geographic distribution :

South-America
(From South of USA down to Argentina)

Common name :

American Trypanosomiases

Disease:

- NL : ziekte van Chagas
- FR : maladie de Chagas
- EN : Chagas disease
- ES : enfermedad de Chagas

Final host:

- Man.
- Wild animals.
- Domestic animals.

Intermediate host:

(Vector)

Blood sucking bug

"Kissing Bugs"

(Triatominae)

Transmission:

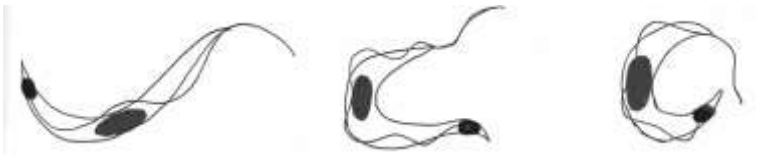
- Metacyclic trypomastigotes from bug's faeces will penetrate the bite wound or the conjunctiva.
- Blood transfusion (fresh blood).
- Congenital transmission.
- (Food or drinks, soiled with the faeces of infected bugs or containing dead bugs?)
- (Cunsumption of undercooked infested animals)

Localisation of the parasite :

- In blood (trypomastigote form)
- Intracellular in tissue (amastigote form)

Diagnostic possibilities:

- Serology (CFT, IFAT, ELISA, agglutination, Dot Blot, …)
- (Examination of lymph node aspirate [or biopsy] for amastigotes).
- Examination of blood for trypomastigotes (acute or congenital phase):
  - in a wet smear (low sensitivity).
  - in a (thin or) thick blood film stained with Giemsa.
  - Woo (micro-haematocrite centrifugation).
  - QBC.
  - Strout concentration technique.
- Blood culture (KIVI : kit for *in vitro* isolation, NNN-medium [Novy-Nicolle-McNeal agar]).
- Detection of specific DNA using PCR
- Xenodiagnosis: searching parasites in feaces of *T. infestans* nyphs (takes up to 60 days for the result).



Morphology of the trypomastigotes (blood after Giemsa staining):

Dimensions : 15-25 µm x 2-6 µm.
Cytoplasm : Pale blue, with a flagellum and an undulating membrane.
Contents : A large central nucleus and a **big** kinetoplast (red).
Characteristics : Usually C, U or S shaped.

Morphology of the amastigotes (biopsy after Giemsa staining):

Dimensions : 4-6 µm.
Cytoplasm : Round, pale blue.
Contents : A central nucleus and a small kinetoplast (red).
Characteristics : Intra or extra- cellular.

Associated biological signs :

- Romaña's sign or chagoma for the acute phase. (occurring in only 5% of the cases)
- Perturbation in hepatical tests for the chronic phase.

Possible confusion with:

- *Leishmania* spp. (amastigotes).
- *Trypanosoma rangeli*.
- Motile bacteria (*Borrelia* spp. or water contaminants) and exflagellation of *Plasmodium* spp. for wet smears.
- *Trypanosoma brucei rhodesiense* (Africa).
- *Trypanosoma brucei gambiense* (Africa).
- Animal's trypanosomiases (*T. brucei brucei*,...)

Remarks :

- In areas where *T. rangeli* (non-pathogenic species transmitted by *Rhodnius* bugs) is found together with *T. cruzi*, these parasites must be differentiated by the size of their kinetoplast.
- The examination for motile trypanosomes must be done as soon as possible: Trypanosomes are unable to survive for more than 15-20 minutes outside of the host's body (glucose consumption). Trypanosomes are also immobilized by sunlight.
- In chronical Chagas disease, the numbers of circulating trypanosomes are too few to be detected using easy techniques. Culture, serology or PCR (or xenodiagnosis) should then be used.
- The amastigotes develop into trypomastigotes which are released into the blood. No multiplication of the parasite occurs in its trypomastigote stage in the blood.
- In a biopsy, it is possible to find amastigotes, but also epimastigotes (intermediary forms between amastigotes and trypomastigotes).

Trypanosoma rangeli

Family : Trypanosomatidae

Class : Mastigophorea

Geographic distribution :

South-America
(Brazil, Venezuela, Colombia, Panama, El Salvador, Ecuador and Guatemala)

Common name :

Disease:

Non pathogenic

Final host:

- Man.
- Wild animals.
- Domestic animals.

Intermediate host:

(Vector)

Blood sucking bug

Rhodnius Bugs

(Triatominae)

Transmission:

- Metacyclic trypomastigotes injected with saliva through the skin when an infested vector takes a blood meal.
- [Metacyclic trypomastigotes, from bug's faeces will penetrate the skin or conjunctiva.]
- Blood transfusion (fresh blood).
- Congenital transmission.
- (Food or drinks, soiled with the faeces of infected bugs or containing dead bugs ?)

Localisation of the trypomastigotes :

In blood (trypomastigote form)

Diagnostic possibilities:

- Identification of trypomastigotes in (Giemsa) stained blood-preparations: species differentiation possible
- Detection of specific DNA using PCR

Morphology of the trypomastigotes (blood after Giemsa staining):

Dimensions : 30 - 35 µm x 2 – 6 µm.

Cytoplasm : Pale blue, with a flagellum and an undulating membrane.

Contents : A large central nucleus and a small kinetoplast (red).

Characteristics : Trypanosomes may show a variety of forms (pleomorphic).

Associated biological signs :

Non pathogenic

Possible confusion with:

- *Trypanosoma cruzi*.
- Motile bacteria (*Borrelia* spp. or water contaminants) and exflagellation of *Plasmodium* spp. for wet smears.
- *Trypanosoma brucei rhodesiense* (Africa).
- *Trypanosoma brucei gambiense* (Africa).
- Animal's trypanosomiases (*T. brucei brucei*,...)

Remarks :

- In areas where *T. rangeli* is found together with *T. cruzi*, these parasites must be differentiated by the size of their kinetoplast.
- Microscopically, the trypanosomes of *T. b. gambiense*, *T. b. rhodesiense* and *T. rangeli* look the same. Clinical status and geographical location will give the species diagnosis.
- In man, *T. rangeli* will only be found in blood (and not in tissue).
- There is no cross immunity between *T. rangeli* and *T. cruzi.*

Leishmania spp.

Family : Trypanosomatidae

Class : Mastigophorea

Geographic distribution :

Various disease forms in southern Europe, tropical and sub-tropical countries.

Common name :

Disease:

- NL : (viscerale, cutane en muco-cutane) leishmaniose
- FR : leishmaniose (viscérale, cutanée et muco-cutanée)
- EN : leishmaniasis (visceral, cutaneous and mucocutaneous)
- ES : leishmaniasis (visceral, cutaneas y mucocutaneas)

Final host:

- Man.
- Various animals (depending on *Leishmania* spp.).

Intermediate host:

(Vector)

Sand flies

Phlebotomus spp. (Old World)

Lutzomya spp. and *Psychodogylus* spp. (New World)

Transmission:

- Metacyclic forms injected with saliva through the skin when an infested vector takes a blood meal.
- Direct contact with leishmaniasis lesions.
- Blood transfusion.
- Crushing an infested vector on (damaged) skin or on the mucosa.
- [Contact with infested vector's faeces: exceptional]

Localisation of the parasite :

Macrophages and other phagocytic cells (amastigotes)

Diagnostic possibilities :

- Microscopic detection of amastigotes in Giemsa stained slides of relevant tissue [lesion's exsudate or biopsy (cutaneous or mucocutaneous leishmaniasis), aspirations from the spleen, liver, bone marrow, lymph gland (visceral leishmaniasis)].
- Isolation of the parasite in culture (Nogushi Wenyon, Tobie's Rabbit Blood, …)
- Montenegro's test (leishmanin test) (for epidemiological studies).
- Serology : antibody detection in serum (IFAT, ELISA, DATS)
- Detection of specific DNA using PCR (diagnosis and (sub-)species differentiation).

Morphology of the amastigotes (Giemsa):

Size : 1,5 - 3 µm x 2,5 – 6,5 µm.

Cytoplasm : Round or oval, pale blue.

Contents : A large central nucleus and a small rod-shaped kinetoplast (red).

Characteristics : the amastigotes may be found extra- or intra-cellularly

Associated biological signs :

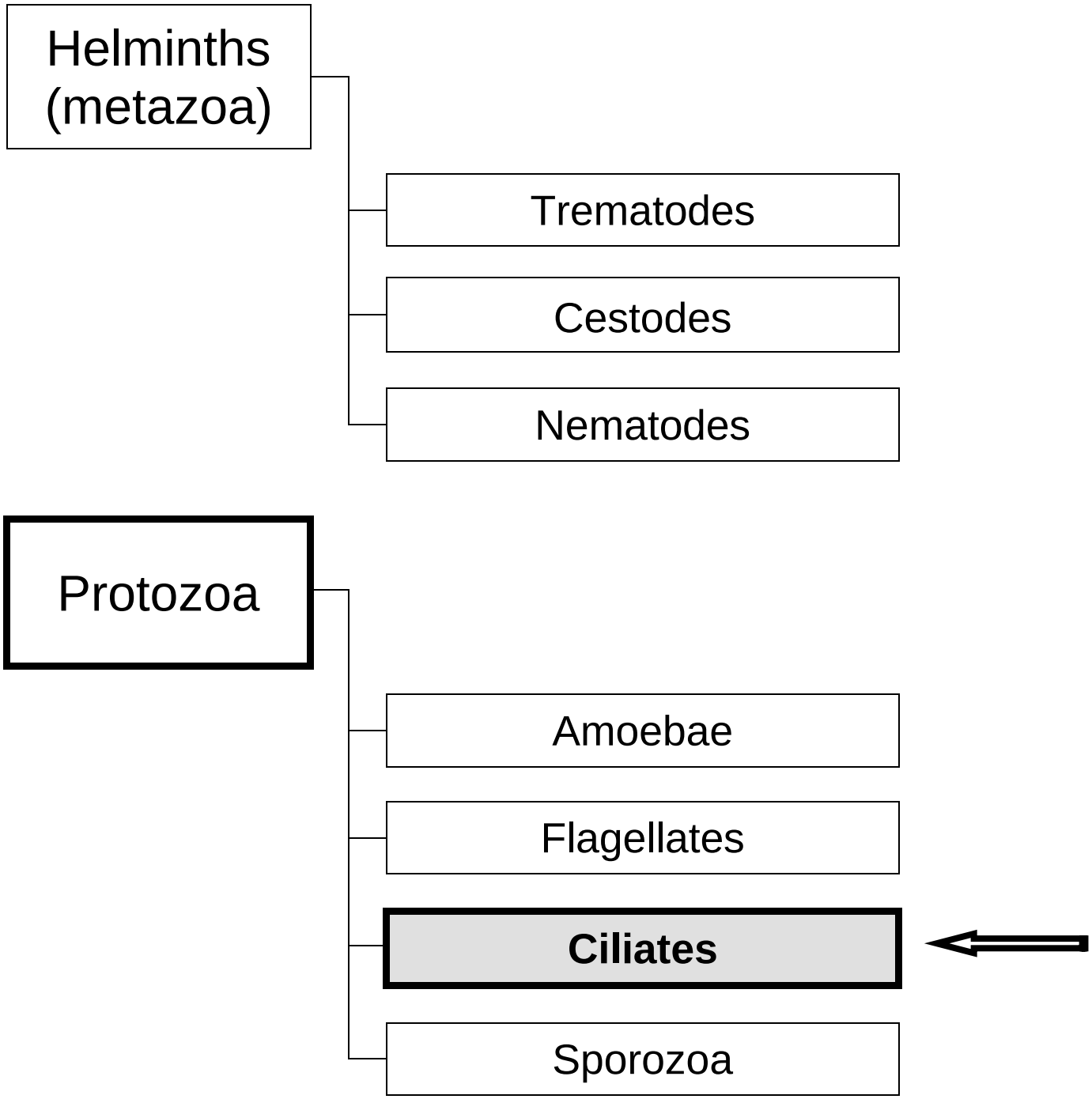
- Visceral leishmaniasis : leucopenia, thrombocytopenia and anaemia
- Perturbation in coagulation and hepatic tests, increases of globulins in blood [IgG] (ESR ↑, positive formol gel test), low serological albumin levels

Possible confusion with:

- Bacteria (diplococci)
- *Trypanosoma cruzi* (Intracellular forms)
- *Toxoplasma gondii*

Remarks :

- Microscopically, the different species of *Leishmania* look the same. Differentiation must be based on monoclonal antibodies or isoenzymatic or molecular analysis. Geographical location may be indicative.
- In case of visceral leishmaniasis, the sensitivity for the parasites detection in Giemsa stained slides depends on the tissue collected: approximately 95% for aspirates from the spleen, 50-85% for bone marrow, 70% for liver biopsies and 65% for lymph gland aspiration. Splenic or hepatic aspirations should be avoided with regard to possible complications during collection.
- Serology is mainly useful for visceral leishmaniasis. The antibodies are even detectable before clinical signs. Sensitivity is good (except for immunocompromised patients). Cross reactions occur with other Trypanosomatidae. Because of the poor antibody response in cutaneous and mucocutaneous leishmaniasis, serological tests are of little value in diagnosis. The formol gel test can prove very useful in cases of visceral leishmaniasis. It shows increases of immunoglobulins (IgG) in the blood (and therefore however is a non-specific test).
- Untreated visceral leishmaniasis always ends in death of the patient.


Helminths (metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Neobalantidium coli

Family : Balantidiae

Class : Listomatea

Geographic distribution :

Rare and Worldwide

Contact with pigs

Common name :

Disease:

- NL : balantidiose
- FR : balantidiase
- EN : balantidiasis
- ES : balantidiasis

Final host :

- Pigs
- Man
- Monkeys, dogs rats

Intermediate host:

Without intermediate host and without vector

Transmission :

Faeco-oral, mature cysts ingestion.

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Trophozoites detection in faeces : direct examination.
- Cysts detection in faeces : direct examination, iodine staining, concentration by sedimentation.
- Trophozoites detection in tissue collected during endoscopy.

Morphology of the trophozoites :

Size : 50-200 µm x 40-70 µm

Nucleus : 2 : one macronucleus (kidney shaped) and one micronucleus.

Motility : In one definite direction. Rapid revolving motility.

Characteristics :
Body surface covered by longitudinal rows of cilia. Cytostome most of the times visible, beating cilia can be seen around the cytostome.

Morphology of the cysts :

Size : 40-65 µm

Morphology : Spherical or oval.

Nucleus : 2 : one macronucleus (kidney shaped) and one micronucleus.

Characteristics :
Thick-walled, cytostome and cilia may be seen in younger cyst.

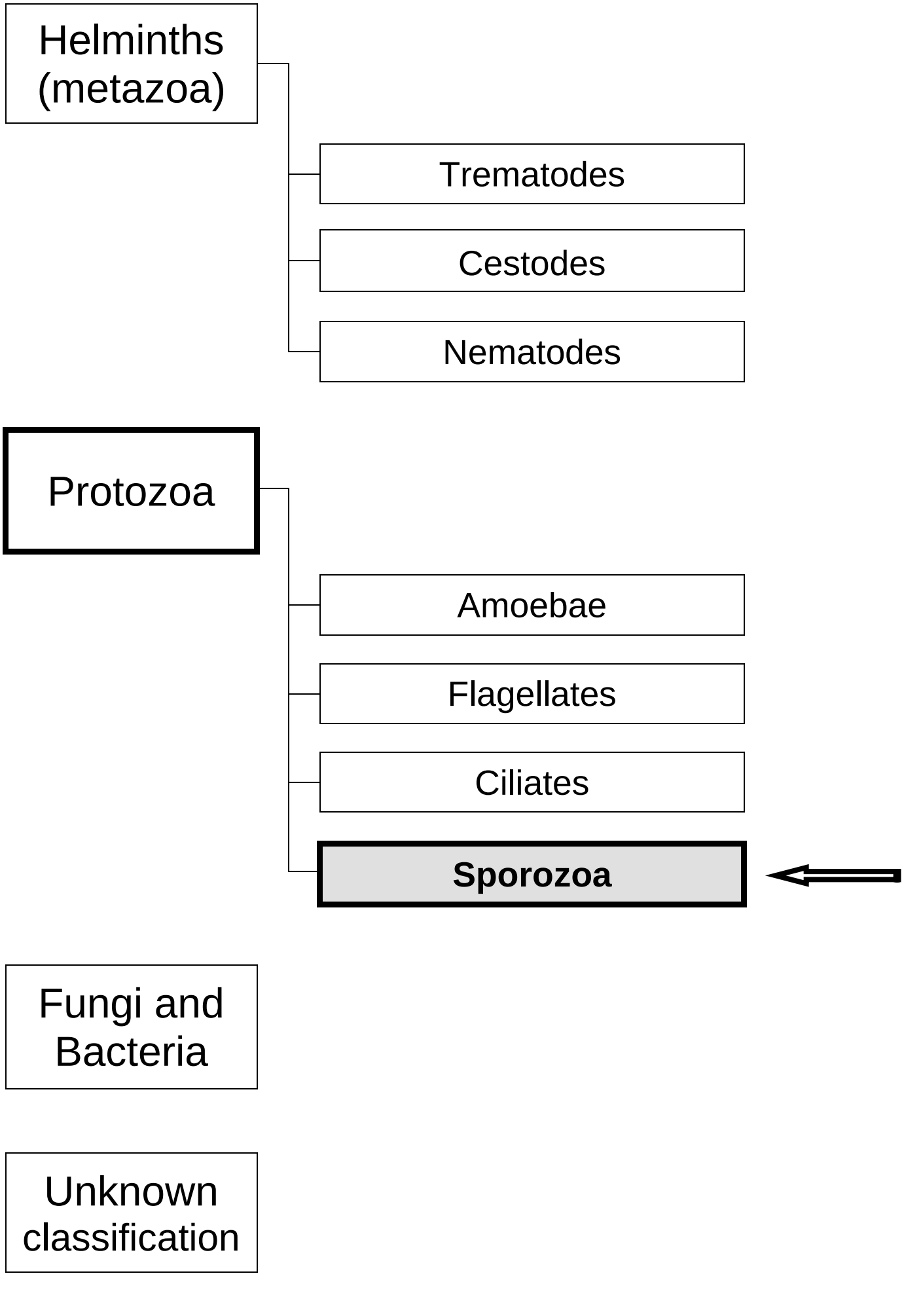
Associated biological signs :

Possible confusion with:

- Plant cells.
- Other non pathogenic ciliates: infusiora type (contamination from the atmosphere).

Remarks :

- *Neobalantidium coli* was formerly known as *Balantidium coli*.
- It is the only ciliate which can parasitize humans. There are a lot of different ciliates in the soil or in the water. Always consider contamination.
- Cysts are found in more formed specimens, trophozoïtes are found mainly in diarrhoeic specimens.
- Trophozoïtes and cysts are excreted irregularly. Several specimens collected at different times may need to be examined.
- Specimens must be examined without delay, otherwise identification of the trophozoïtes becomes impossible because they lose their motility.
- Iodine stain will immediately kill any trophozoïtes.
- *Neobalantidium coli* usually remains confined to the intestinal lumen. In some rare cases, the trophozoites invade the intestinal mucosa, or (exceptionally) through the bloodstream extra intestinal sites (liver, lungs,…).
- This parasite is more frequently found in patients working at pig-farms.


Helminths (metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Plasmodium falciparum

Family : Plasmodiidae

Class : Haemosporidea

Geographic distribution :

Tropical and subtropical regions
- America
- Africa
- Asia (airports!!!)

Common name :
- Malaria
- Paludism

Disease:
- NL : kwaardaardige tertiana, pernicieuze koorts
- FR : fièvre tierce maligne, fièvre pernicieuse
- EN : falciparum malaria
- ES : tertiana maligna, fiebre perniciosa

Final host:
- Man

Intermediate host:

(Vector)

Female mosquitoes

Anopheles spp.

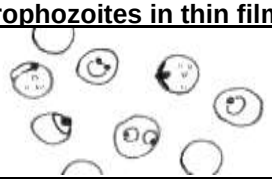
Transmission:
- Sporozoites inoculation during a blood meal by a malaria infested vector.
- Blood transfusion.
- Congenital transmission.

Localisation of the parasite :
- Intracellular in parenchymal cells of the liver.
- Intracellular in red blood cells.

Diagnostic possibilities:
- Blood parasites detection :
  - In a thin blood film (species differentiation and quantification).
  - In a thick blood film (pre-differentiation of species and quantification).
  - In QBC (pre-differentiation of species and quantification).
- Serology
  - *Plasmodium*'s specific Ag detection : HRP-II, Aldolase, pLDH (rapid tests)
  - *Plasmodium*'s specific Ab detection : IFA, ELISA, … (epidemiology)
- Detection of specific DNA using PCR
- (Malaria pigment and/or RBC alterations detection with an haematological analyser)

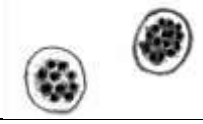
Trophozoites in thick film :

Trophozoites in thin film:



Schizonts in thick film :

Schizonts in thin film:



Gametocytes in thick film:

Gametocytes in thin film:

Main characteristics in thick film:
- Usually only trophozoites and gametocytes seen (except for severe infections).
- Small ring or comma forms, often 2 chromatin dots.
- Banana shaped gametocytes (if present).
- Uniform image.

Main characteristics in thin film :
- Infected erythrocyte has normal size and shape.
- Usually only trophozoites and gametocytes seen (except for severe infections)
- Small ring forms, trophozoites may lie on RBC membrane (accolé forms or marginal forms)
- Polyparasitism common.
- Banana shaped gametocytes (if present).
- Maurer's clefts possible (few large pink granules in older infected RBC; only at pH=8).

Main associated biological signs :
- Fever.
- Anaemia (haemolytic).
- Trombocytopenia.
- Hypoglycaemia.
- High (direct) bilirubin and LDH level.
- Low haptoglobin level.

Possible confusion with:
- Blood platelets
- Other *Plasmodium* spp.
- Howell-Jolly bodies
- *Babesia* spp. and *Theileria* spp.
- *Toxoplasma gondii* (~ gametocytes)

Remarks :
- In a thick blood film, the uniform aspect of *Plasmodium falciparum* may help to make the differential diagnosis.
- Exceptionally, in heavy infection, no parasites will be found (*Plasmodium falciparum* infested RBCs adhere to endothelium in organ capillaries = sequestration)
- The presence of schizonts of *P. falciparum* in the periferal bloodstream and/or the presence of hemozoïne in WBCs indicate severe infestations.
- Gametocytes are not seen early in the infection (about 10 days after initial infection), so their absence from blood films should not exclude a diagnosis. Gametocytes can exceptionally be round or exhibit exflagellation!
- Parasitemia up to 50% of RBCs infested and more (2.500.000 parasites/µl of blood or more).

Plasmodium vivax

Family : Plasmodiidae

Class : Haemosporidea

Geographic distribution :

(at least 16°C isotherm during the hot season)

- America
- Asia
- Africa
- …

Common name :

- Malaria
- Paludism

Disease:

- NL : goedaardige tertiana
- FR : fièvre tierce bénigne
- EN : vivax malaria
- ES : tertiana benigna

Final host:

- Man

Intermediate host:

(Vector)

Female mosquitoes

Anopheles spp.

Transmission:

- Sporozoites inoculation during a blood meal by a malaria infested vector.
- Blood transfusion.
- Congenital transmission.

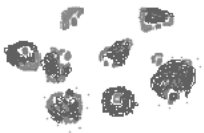
Localisation of the parasite :

- Intracellular in parenchymal cells of the liver (hypnozoïtes possible).
- Intracellular in red blood cells.

Diagnostic possibilities:

- Blood parasites detection :
  - in thin blood film (species differentiation and quantification).
  - in thick blood film (pre-differentiation of species and quantification).
  - in QBC (pre-differentiation of species and quantification).
- Serology
  - *Plasmodium*'s specific Ag detection : Aldolase, pLDH (rapid tests)
  - *Plasmodium*'s specific Ab detection : IFA, ELISA, … (epidemiology)
- Detection of specific DNA using PCR
- (Malaria pigment and/or RBC alterations detection with an haematological analyser)

Trophozoites in thick film :



Trophozoites in thin film:

Schizonts in thick film :

Schizonts in thin film:

Gametocytes in thick film:

Gametocytes in thin film:

Main characteristics in thick film:

- All stages may be found in the same film.
- Typical amoeboid old trophozoites.
- Mature schizonts with 16 merozoites (or more).
- Small pink dots (Schüffner's stippling) in "ghost" of RBC (only for old forms).

Main characteristics in thin film :

- Large and/or irregular host cells.
- All stages may be found in the same film.
- Typical amoeboid old trophozoites.
- Mature schizonts with 16 merozoites (or more).
- Fine Schüffner's stippling (only for old forms).

Main associated biological signs :

- Fever.
- Thrombocytopenia.

Possible confusion with:

- Platelets
- Other *Plasmodium* spp.
- Howell-Jolly bodies
- *Babesia* spp. and *Theileria* spp.

Remarks :

- Relapses are a feature of *P. vivax* malaria, due to the delayed release of merozoites from liver cells (hypnozoites).
- In a thick blood film, the cloud-like aspect of *Plasmodium vivax* may help to make the differential diagnosis.
- Rarely found in Duffy negative population (Central Africa). Duffy = blood-group Ag which *P. vivax* needs to attach to and invade RBC.
- All stages (trophozoites, schizonts and gametocytes) may be found at the same time in the peripheral blood.
- Parasitemia up to 4% of RBCs infested (or 200.000 parasites/µl of blood).

Plasmodium ovale

Family : Plasmodiidae

Class : Haemosporidea

Geographic distribution :

- Tropical and sub-tropical Africa
- West Pacific
- South America (small focusses)

Common name :

- Malaria
- Paludism

Disease:

- NL : goedaardige tertiana
- FR : fièvre tierce bénigne
- EN : ovale malaria
- ES : fiebre tertiana

Final host:

- Man

Intermediate host:

(Vector)

Female mosquitoes

Anopheles spp.

Transmission:

- Sporozoites inoculation during a blood meal by a malaria infested vector.
- Blood transfusion.
- Congenital transmission.

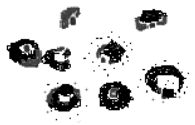
Localisation of the parasite :

- Intracellular in parenchymal cells of the liver (hypnozoïtes possible).
- Intracellular in red blood cells.

Diagnostic possibilities:

- Blood parasites detection :
  - In thin blood film (species differentiation and quantification).
  - In thick blood film (pre-differentiation of species and quantification).
  - In QBC (pre-differentiation of species and quantification).
- Serology
  - *Plasmodium*'s specific Ag detection : Aldolase, pLDH (rapid tests)
  - *Plasmodium*'s specific Ab detection : IFA, ELISA, … (epidemiology)
- Detection of specific DNA using PCR
- (Malaria pigment and/or RBC alterations detection with an haematological analyser)

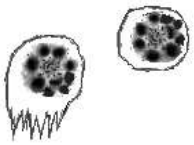
Trophozoites in thick film :



Trophozoites in thin film:

Schizonts in thick film :

Schizonts in thin film:



Gametocytes in thick film:

Gametocytes in thin film:

Main characteristics in thick film:

- All stages may be found in the same film.
- Parasites quite small and compact.
- Mature schizonts usually contain 8 merozoites.
- Large pink/red dots (Schüffner's stippling) in "ghost" of RBC.

Main characteristics in thin film :

- Large host cells, often oval, with torn jagged edges.
- All stages may be found in the same film.
- Parasites quite small and compact.
- Mature schizonts usually contain 8 merozoites.
- Prominent pink/red dots (Schüffner's stippling) in the infected erythrocyte.

Main associated biological signs :

- Fever.

Possible confusion with:

- Platelets
- Other *Plasmodium* spp.
- Howell-Jolly bodies
- *Babesia* spp. and *Theileria* spp.

Remarks :

- Relapses are a feature of *P. ovale* malaria, due to the delayed release of merozoïtes from liver cells (hypnozoïtes).
- All stages (trophozoites, schizonts and gametocytes) may be found at the same time in the peripheral blood.
- Parasitemia up to 4% of RBCs infested (or 200.000 parasites/µl of blood).

Plasmodium malariae

Family : Plasmodiidae

Class : Haemosporidea

Geographic distribution :

More or less cosmopolite

- Common in Africa and Asia
- Less common in America

Common name :

- Malaria
- Paludism

Disease:

- NL : goedaardige quartana
- FR : fièvre quarte bénigne
- EN : malariae malaria
- ES : la cuartana

Final host:

- Man

Intermediate host:

(Vector)

Female mosquitoes

Anopheles spp.

Transmission:

- Sporozoites inoculation during a blood meal by a malaria infested vector.
- Blood transfusion.
- Congenital transmission.

Localisation of the parasite :

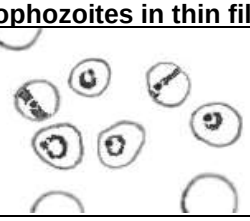
- Intracellular in parenchymal cells of the liver.
- Intracellular in red blood cells.

Diagnostic possibilities:

- Blood parasites detection : - In thin blood film (species differentiation and quantification).
  - In thick blood film (pre-differentiation of species and quantification).
  - In QBC (pre-differentiation of species and quantification).
- Serology : - *Plasmodium*'s specific Ag detection : Aldolase, pLDH (rapid tests)
  - *Plasmodium*'s specific Ab detection : IFA, ELISA, … (epidemiology)
- Detection of specific DNA using PCR
- (Malaria pigment and/or RBC alterations detection with an haematological analyser)

Trophozoites in thick film ;

Trophozoites in thin film:

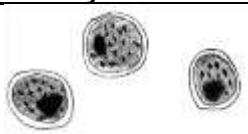


Schizonts in thick film :

Schizonts in thin film:

Gametocytes in thick film:

Gametocytes in thin film:



Main characteristics in thick film:

- All stages may be found in the same film.
- Mature schizonts contain 6-12 merozoites, arranged in rosette formation around a clump of pigment.
- Parasites small, compact, densely stained.
- Pigment already present in early stages.

Main characteristics in thin film :

- Infected RBCs are normal or smaller.
- All stages may be found in the same film.
- Parasites small, compact, densely stained.
- Mature schizonts contain 6-12 merozoites, arranged in rosette formation around a clump of pigment.
- Pigment already present in early stages.
- Trophozoïtes in characteristic band form (rare).

Main associated biological signs :

- Fever

Possible confusion with:

- Platelets
- Other *Plasmodium* spp.
- Howell-Jolly bodies
- *Babesia* spp. and *Theileria* spp.

Remarks :

- Parasitaemia is usually low (less than 1% of RBCs infested or 50.000 parasites/µl of blood).
- All stages (trophozoites, schizonts and gametocytes) may be found at the same time in the peripheral blood.
- In a thick blood film, the "dirty" aspect of *Plasmodium malariae* may help to make the differential diagnosis.
- *Plasmodium knowlesi*, a malaria parasite infesting monkeys and sporadically humans can be found in the area of Malaysia and Borneo and more sporadically in Thailand, Myanmar, the Philippines and Singapore. It has the same morphology as *P. malariae*, but with a significantly higher parasitaemia. Because *P. knowlesi* replicates every 24h, prompt effective treatment is essential. **All patients coming from Southeast Asia presenting "*P. malariae*" hyperparasitaemia should receive intensive management as appropriate for severe falciparum malaria.**

Characteristics of *Plasmodium* species in thin blood films

Characteristics	*Plasmodium falciparum*	*Plasmodium vivax*	*Plasmodium malariae*	*Plasmodium ovale*
Incubation period	7 - 21 days Mean = 12	8 - 31 days Mean = 14	19 - 37 days Mean = 28	11 – 16 days
Hypnozoites	No	Yes	No	Yes
Relapse	No, but sometimes recrudescence up to 1 year	Yes, ± 6 months to 4 years	Possible after a long period of time (recrudescence up to 52 years)	Possible, but most of the time natural suppression
Asexual cycle in blood	24-48 hours	48 hours	72 hours	48 hours
Size of infected erythrocyte	Normal (infects all RBCs)	Large and often pale blue staining (infects young RBCs)	Normal or smaller (infects old RBCs)	Large, normal or smaller; oval with torn jagged edges
Dots in the infected erythrocyte	Maurer's clefts (pH=8): in some infected RBCs with mature trophozoites	Schüffner's dots : small pink dots found in some infected RBCs with mature trophozoites	No	Schüffner's dots : large red dots practically always present
Stages found	Most of the time, only trophozoites and/or gametocytes. Exceptionally, schizonts and young gametocytes (severe infections)	All stages may be found	All stages may be found	All stages may be found
Parasite density	Up to 2.500.000/µl and more	Maximum 200.000/µl (4%)	Maximum 50.000/µl (1%)	Maximum 200.000/µl (4%)
Young trophozoite :				
Cytoplasm	Small, fine pale ring Occasional "accolé" forms	Quite big ring	Compact ring	Quite compact
Chromatin	1 to 2 red dots	Quite big, sometimes 2 dots	Generally 1dot	1 dark dot
Vacuole	Visible	Big	Not or barely visible	Not or barely visible
Pigment	No	No	Sometimes	No
Polyparasitism	Often	Sometimes	Usually not	Usually not
Mature trophozoite :				
Cytoplasm	Little amoeboïd, most of the time lying on the red cell membrane	Amoeboid, fragmented, may almost fill the RBC	Compact, sometimes amoeboïd ; Typical band form	Little amoeboïd, do not fill the RBC
Chromatin	1 to 2 medium size red dots	1 or 2 quite big dots	1 to 2 big dots	1 tot 2 dark black dots
Vacuole	1 or more, variable size	1 or more big vacuoles	Not visible	Small, not all the times visible
Pigment	Exceptionally, 1 black dot	Small, brown, scattered, sometimes 1 compact mass	Yes (big, black mass)	Very rare
Young schizont :				
Cytoplasm	Irregular, few	Large, irregular	Compact	Compact
Chromatin	2 – 4	2 or more, variable size	2 – 4	2 – 4
Vacuole	No	Sometimes up to 4 small vacuoles	NO	NO
Pigment	Clumped in one mass	Dark, scattered	Dark, scattered on the parasite	Dark, scattered on the parasite

	Plasmodium falciparum	***Plasmodium vivax***	***Plasmodium malariae***	***Plasmodium ovale***
Mature schizont :				
Cytoplasm	2/3 of the RBC	May almost fill the RBC	May almost fill the RBC	Do not fill the RBC
Chromatin	8 – 24 or more	12 – 24, most of the time 16	6 -12, most of the time 8, clustered around	4 – 16
Pigment	Single dark mass	Concentrated in 1 or 2 masses	Compact mass, sometimes central	Concentrated in 1 or 2 masses
Macrogametocyte (female gametocyte) :				
Cytoplasm	Pale blue, banana shaped, exceptionally round, RBC most of the time not visible	Pale blue, round of oval, may almost fill the RBC	Pale blue, round, may almost fill the RBC	Pale blue, round, do not fill the RBC
Chromatin	1 well defined mass	1 well defined mass	1 well defined mass	1 well defined mass
Pigment	Dark, scattered rice-grain like, lying on the nucleus	Brown granualtions, scattered on the parasite	Dark granulations, scattered on the parasite	Dark granulations, scattered on the parasite
Microgametocyte (male gametocyte) :				
Cytoplasm	Pink-blue, banana shaped, exceptionally round, RBC most of the time not visible	Pink-blue or colorless, round or oval; may almost fill the RBC	Pink-blue or colorless, round or oval; may almost fill the RBC	Pink-blue or colorless, round or oval; fillnot the RBC
Chromatin	1 big diffuse mass	1 big, diffuse chromatin mass	1 big, diffuse mass	1 big, diffuse mass
Pigment	Dark, scattered rice-grain like, scatered on the nucleus	Brown-black, scaterred on the parasite	Dark, brown-black, scaterred on the parasite	Dark, scaterred on the parasite
Main characteristics :				
	➢ Infected erythrocytes have normal size. ➢ Only trophozoites and gametocytes usually seen (except in severe infections) ➢ Small ring forms, trophozoites may lie on red cell membrane (marginal forms) ➢ Polyparasitism common. ➢ Banana shaped gametocytes. ➢ Maurer's clefts, few large pink granules can be found in some infected red cells (pH=8). ➢ Often very high parasite density : can be over 50%	➢ Enlarged or irregular host cell. ➢ All stages usually seen in the same film. ➢ Typical amoeboid old trophozoites. ➢ Mature schizonts with 16 merozoites (or more). ➢ Fine Schüffner's stippling (only for old forms). ➢ Medium parasite density : up to 4%.	➢ Infected cells are normal or smaller. ➢ All stages usually seen in the same film. ➢ Parasites small, compact, densely stained. ➢ Mature schizonts contain 8 merozoites, arranged in rosette formation around a clump of pigment. ➢ Pigment early present. ➢ Trophozoites in the characteristic band form (rare) ➢ Low parasite density: up to 1%	➢ Infected cells enlarged, oval, with torn jagged edges. ➢ All stages usually seen in the same film. ➢ Parasites quite small and compact. ➢ Mature schizonts contain 8 merozoites. ➢ Prominent pink/red dots (Schüffner's stippling) in the infected erythrocyte. ➢ Medium parasite density: up to 4%

Characteristics of *Plasmodium* species in thick blood films

Characteristics	*Plasmodium falciparum*	*Plasmodium vivax*	*Plasmodium malariae*	*Plasmodium ovale*
Dots surrounding parasite	No	Schüffner dots: fine stipplings in "ghost" of host red cells	No	Schüffner dots: big stipplings in "ghost" of host red cells
Stages found	Most of the time, only trophozoites and/or gametocytes. Exceptionally schizonts (severe infections)	All stages may be found	All stages may be found	All stages may be found
Trophozoites :				
Size	Small to medium	Small to big	Small	Small to medium
Form	Ring and comma forms common	Broken ring or irregular form	Ring or round, compact	Ring or round, compact
Cytoplasm	Regular, fine to fleshy	Irregular or fragmented	regular, dense	Fairly regular
Chromatin	1 to 2 dots	Quite big, occasionally 2 dots	Single large dot	1 dense dot
Pigment	Exceptionally compact mass	Scattered, fine	Scattered, coarse	Scattered, big
Schizonts :				
Size	Small, compact	Big	Small, compact	Medium
Chromatin	8 to 24 or more	12 to 24, most of the times 16	6 to 12, most of the times 8	4 to 16, most of the times 8
Pigment	Single dark mass	Diffuse mass	Concentrated mass, occasionally in the center	Concentreted
Gametocyte :				
Form	Banana shaped, exceptionally round	Round, big	Round, compact	Round, medium size
Chromatin	1 well defined mass	1 well defined mass	1 well defined mass	1 well defined mass
Pigment	Scattered, coarse rice grain like	Scattered, fine	Scattered, big mass	Scattered big dots
Main characteristics :				
	➢ Only trophozoites and gametocytes usually seen (except in severe infections). ➢ Small ring or comma forms, often 2 chromatin dots. ➢ Banana shaped gametocytes. ➢ Uniform image.	➢ All stages may be seen in the same film. ➢ Typical amoeboid old trophozoites. ➢ Mature schizonts with 16 merozoites (or more). ➢ Small pink dots (Schüffner's stippling) in "ghost" of red cells (only for old forms).	➢ All stages may be seen in the same film. ➢ Mature schizonts contain 6-12 merozoites, arranged in rosette formation around a clump of pigment. ➢ Parasites small, compact, densely stained. ➢ Pigment early present.	➢ All stages may be seen in the same film. ➢ Parasites quite small and compact. ➢ Mature schizonts contain 8 merozoites. ➢ Large pink/red dots (Schüffner's stippling) in "ghost" of red cells.

Babesia *spp*.

Family :

Babesiidae

Class :

Piroplasmidea

Geographic distribution :

Cosmopolitic **?**

America, Europe, Asia, Africa **?**

Common name :

Pathology :

- NL: Babesiose, piroplasmose
- FR: Babésiose, piroplasmose
- EN: Babesiosis, piroplasmosis
- ES: Babesiose, piroplasmose

Final host :

- Various domestic animals.
- Various wild animals.
- (Man).

Intermediate host :

Vector and reservoir :

Hard and soft ticks

Ixodes spp.(hard)
Ornithodoros spp.*(*soft*)*
…

Transmission :

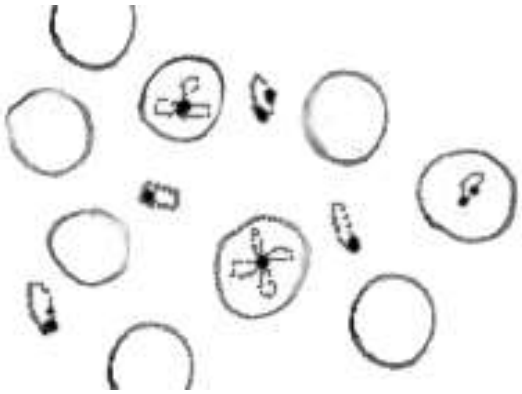
- Trophozoites injected with saliva through the skin when an infested vector takes a blood meal.
- Blood transfusion.
- [Congenital transmission?].

Localisation of parasites :

Intracellular in red blood cells (and monocytes) AND extracellular in blood.

Diagnostic possibilities :

- Parasite detection in the blood by microscopy (thick or thin film).
- Serology : Detection of specific antibodies IgM and/or IgG (IFAT, ELISA,…).
- Detection of specific DNA using PCR.
- [Detection of parasites after inoculation of the animal].



Morphology of trophozoites :

(thin film stained with Giemsa)

Dimensions :	Variable, 1 to 5 µm.
Localisation :	1 tot 4 trophozoites per red blood cell, extracellular
Morphology :	Round, oval, pear-shaped
Cytoplasm :	Stained blue, no pigment.
Nucleus :	1 to 4 small red nuclei
Characteristics :	Multiplication by budding, forming clover-shaped structures with 2, 3 and eventually 4 leaves (tetrade or **Maltezer cross**). Never pigment. Extracellular clumps of parasite.

Associated biological signs :

- Fever.
- Anaemia.
- Thrombocytopenia.

Possible confusion with :

- Trophozoites *of Plasmodium* spp. (mostly *falciparum*).
- Blood platelets.
- Howell-Jolly bodies.
- *Theileria* spp.

Remarks :

- More than 110 different species of *Babesia* are identified, but only some of them infest humans : *Babesia microti*, *Babesia* WA, *Babesia* MO1 (America), *Babesia divergens*, *Babesia bovis* (Europe), …
- Most of human infestations occur asymptomatically, taken into account the results of a serological surveillance done in an endemic region. Immuno-depression, splenectomy and age make man more susceptible to Babesiosis. *Babesia divergens* (Europe) is the most dangereous parasite, but luckily also the rarest one.
- Differential diagnosis between trophozoites of *Plasmodium falciparum* and *Babesia* spp. is difficult. This could be the reason why in malaria-endemic regions *Babesia* is under- or misdiagnosed.
- Coinfection with *Borrelia burgdorferi* (Lyme disease, identical transmission, frequent coinfestation of vectors and experimental transmission of both agents possible during the same blood meal) is well described in America (upto 89 % of the cases).
- Another bacterial family (*Ehrlichia* spp.) (co-)transmitted by ticks in the UK and Japan causes Human Granulocytic Ehrlichiosis (HGE) or Human Granulocytic Anaplasmosis, which is a also a very rare disease. Diagnosis by serology.
- Some species of *Babesia* can be transmitted from a female tick to its offspring (transovarian) before migrating to the salivary glands for feeding. Thus the vertebrate host is not indispensable for the survival of the parasite (we can not talk about a 'definitive' vertebrate host). *B. microti*, the most common variety of *Babesia* in humans however, has not been shown to transmit transovarially.
- Another family of parasites with similarities (*Theileria* spp.) infests animals. Up to now, human infections have not been described.

Toxoplasma gondii

Family : Sarcocystiidae

Class : Coccidea

Geographic distribution :

Worldwide

Common name :

Disease:

- NL : toxoplasmose
- FR : toxoplasmose
- EN : toxoplasmosis
- ES : toxoplasmosis

Final host:

- Felidae

Intermediate host:

- Various animals
- Man

Transmission:

- Transparenteral (blood transfusion and organ transplantation).
- Ingestion of sporulated oocysts [by way of cats].
- Ingestion of meat contamined by bradyzoites (tachyzoites) [by way of sheep].
- Ingestion of bradyzoites after meat manipulation.

Localisation of the parasite :

Intracellular, in internal organs

Diagnostic possibilities:

- Serology (IgM, IG G, Ig A, [Ig E] . Especially in detecting congenital infections in utero.
- Tachyzoites detection in Giemsa stained preparations (various specimens : lymph nodes or bone marrow aspirates, CSF, placenta, bronchoalveolar lavage,…).
- Bradyzoites or tachyzoites detection in histological preparation.
- (Parasites detection after culture or animal inoculation)
- Detection of specific DNA using PCR.
- X-ray, scanner, …

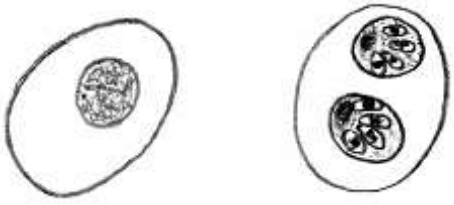
Morphology of the trophozoites (man):

(Giemsa)

5 - 12 µm x 2 – 4 µm, intracellular (Round or ovale) or extracellular (water-drop shaped). Pale blue cytoplasm and central red nucleus.

Morphology of the cysts (man-animals) :

(Mainly in brain or muscular tissues). Up to 200 µm, spherical or oval, with a thick shell. The cyst contains 50 to 3.000 **bradyzoites**.



Morphology of the oocysts (cat faeces) :

10-15 µm, spherical or oval, non sporulated oocyst contains a granular masse. Sporulated oocyst contains 2 **sporocysts** containing 4 **sporozoites**

Main associated biological signs :

- Various
- (immunocompromission)

Possible confusion with :

- *Leishmania* spp., *Pneumocystis* spp. and *Sarcocystis* spp.
- *Plasmodium falciparum* (gametocytes).

Remarks :

- Parasitaemia is usually low.
- For immunocompromised patients, it is possible to find tachyzoites in peripheral blood monocytes.
- Serological tests may help for immunocompromised patients, but the interpretation of tests results is often difficult.

Sarcocystis spp.

Family : Sarcocystiidae

Class : Coccidea

Geographic distribution :

Worldwide

Common name :

Disease:

- NL : (Intestinale of musculaire) sarcocystose
- FR : Sarcocystose (intestinale ou musculaire)
- EN : (Intestinal or muscular) Sarcocystosis
- ES : Sarcocystosis intestinal or muscular

Final host:

- Carnivorous
- Omnivorous
- (Man)

Intermediate host:

- Pigs
- Herbivorous
- (Man)

Transmission:

- Intestinal form : Ingestion of meat contamined by cysts [intermediate host].
- Muscular form : faeco-oral : ingestion of sporocysts [final host].

Localisation of the parasite :

- Intestinal form : Schizogony in epithelial cells of the intestine. Oocysts excreted in the faeces.
- Muscular form : Schizogony in epithelial cells of the blood vessel, muscular cysts formation.

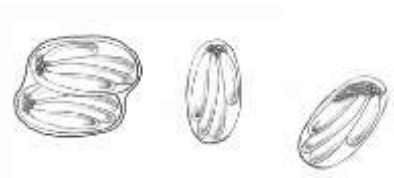
Diagnostic possibilities:

Intestinal form :

- Oocysts detection in faeces : Direct examination, concentration by sedimentation or by flotation.
- Detection of specific DNA using PCR.

Muscular form :

- Cysts (sarcocysts) identification in muscular biopsy.



Morphology of the oocysts :

Faeces, direct examination

Size : 11-17 µm x 10-16 µm

Morphology : Oval or variable.

Contents : 2 sporocysts with 4 banana shaped sporozoites inside

Characteristics :

Sarcocystis oocysts are fragile. Most of the times, the sporocysts are found freely in faeces. Sporocysts in faeces are always sporulated and contain a refringent mass.

Main associated biological signs :

- Watery diarrhoea without blood
- Often hypereosinophilia.
- Often presence of Charcot-Leyden crystals in the faeces

Possible confusion with :

- *Giardia lamblia* (cysts).
- Animal coccidians (oocysts in transit).

Remarks :

- The number of parasites found is usually very low, rendering concentration-techniques quite useful. For the intestinal form, oocysts can be detected in the faeces starting from about two weeks after infestation
- Muscular or intestinal infestation are rarely serious and often asymptomatic. This parasite is more an opportunist.
- Humans may serve as either final (Intestinal infection with *Sarcocystis bovi-hominis* (herbivorous as intermediate host) or *Sarcocystis sui-hominis* (pigs as intermediate host)) or intermediate host (Muscular infection with different species of S*arcocystis* with a variety of different final hosts).
- Oocysts present in human faeces are always sporulated and are not infectious to humans.
- During the short blood part of their life cycle (muscular form only), rare sporozoites may be found in blood (to be differentiated from *Toxoplasma* spp. or gametocytes of *Plasmodium falciparum.*
- *Isospora hominis*, is the old name for the human intestinal form of *Sarcocystis* spp*.*
- When stained with the negative staining technique of Heine, the oocysts (and sporocysts) show as non-stained, strongly refractile structures on a pink to purple background.
- As the oocysts (and sporocysts) are acid-fast structures, they will stain pink on a blue background, when using the (modified) Ziehl-Neelsen technique. Some of the oocysts (and/or sporocysts) will not stain however.
- The oocysts (and sporocysts) can also be detected by their autofluorescence. They light up violet at a wavelenghth of 365 nm, green at 405 nm and blue to violet at 436 nm. This technique can only be used on thin preparations of untreated faeces or faeces treated with SAF or 10% formaline solution (if desired after concentration using sedimentation).

(Cysto)isospora belli

Family : Eimeriidae

Class : Coccidea

Geographic distribution :

Worldwide

Common name :

Disease:

- NL : isosporose
- FR : isosporose
- EN : isosporosis
- ES : isosporosis

Final host:

- Man

Intermediate host:

Without intermediate host and without vector

Transmission:

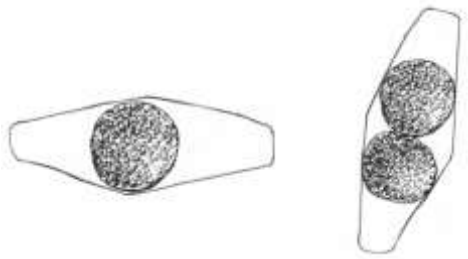
Oral ingestion of sporulated oocysts.

Localisation of the parasite :

Intracellular, schizogony in epithelial cells of the intestine.

Diagnostic possibilities :

- Oocysts detection in faeces : direct examination, concentration by sedimentation.
- Oocysts detection in duodenal aspirate (or using a Stringtest (Enterotest®) or in duodenal biopsy : direct examination.
- Detection of specific DNA using PCR.



Morphology of the oocysts :

Faeces, direct examination

Size : 25-33 µm x 12-16 µm

Morphology : Ellipsoidal.

Contents : 1 then 2 non sporulated sporoblasts (granular mass)

Characteristics : Fine and transparent shell.

Main associated biological signs :

- Watery diarrhoea without blood.
- Often eosinophilia.
- Often presence of Charcot-Leyden crystals in the faeces.

Possible confusion with :

- Animal coccidians (oocysts in transit).

Remarks :

- Human infection with *Isospora belli* is rarely serious and often asymptomatic. This parasite is more an opportunist.
- Oocysts of *Isospora belli* are difficult to distinguish with a 10x objective (they are too transparent). The examination should be done with a 40x objective.
- Because the oocysts may be passed in small amounts and intermittently, repeated stool examinations and concentration procedures are recommended.
- Oocysts of *Isospora belli* are unsporulated at the time of excretion and do not become infective until sporulation is completed. They need a certain period of time for "maturation" in the environment before becoming infectious. This maturation depends on climatological factors (temperature, humidity) but is at least some days. Once sporulated, the oocyst contains two sporocysts, each containing four sporozoïtes.
- *Isospora belli* oocysts are quite resistant to disinfectants (cresol, iodine, chlorine,…). They are killed by ammonium or formalin.
- When stained with the negative staining technique of Heine, the oocysts show as non-stained, strongly refractile structures on a pink to purple background. Their morphology will be less typical though.
- Most of *Isospora belli* oocysts are acid fast (pink to red when coloured with Ziehl-Neelsen). Some oocysts may stain palely or will be colourless but the typical ellipsoidal shape will still be visible.
- The oocysts can also be detected by their autofluorescence. They light up violet at a wavelenghth of 365 nm, green at 405 nm and blue to violet at 436 nm. This technique can only be used on thin preparations of untreated faeces or faeces treated with SAF or 10% formaline solution (if desired after concentration using sedimentation).
- Some authors (Barta et. al., 2005) insist that all tetrasporozoic, diplosporocystic oocysts without Stieda-bodies, amongst which *Isospora belli*, be transferred to the genus *Cystoisospora* (Fam. Sarcocystidae), infecting mammals, and that the genus *Isospora* (Fam. Eimeriidae) is reserved for all tetrasporozoic, diplosporocystic oocysts with Stieda-bodies, infecting principally birds. They hereby ask for this parasite to be called *Cystoisospora belli*.

Cyclospora cayetanensis

Family : Eimeriidae

Class : Coccidea

Geographic distribution :

Worldwide

Common name :

Disease:

- NL : cyclosporose
- FR : cyclosporose
- EN : cyclosporosis
- ES : cyclosporosis

Final host:

- Man
- Other animals ?

Intermediate host:

Without intermediate host and without vector

Transmission:

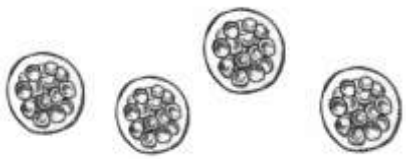
Oral ingestion of sporulated oocysts.

Localisation of the parasite :

Intracellular, schizogony in epithelial cells of the intestine.

Diagnostic possibilities :

- Oocysts detection in faeces : direct examination, concentration by sedimentation.
- Oocysts detection in duodenal aspirate (or using a Stringtest (Enterotest®) or in duodenal biopsy : direct examination.
- Detection of specific DNA using PCR.



Morphology of the oocysts :

Faeces, direct examination

Size : 8 -10 µm.

Morphology : Round.

Contents : Numerous small spherical elements (“morula”)

Characteristics :
Thin shell but well visible.
Transparent, “lens” aspect.

Main associated biological signs :

- Watery diarrhoea without blood.

Possible confusion with :

- Animal coccidians (oocysts in transit).
- *Cryptosporidium* spp. (Ziehl-Neelsen).
- Yeast (Ziehl-Neelsen).

Remarks :

- Human infection with *Cyclospora cayetanensis* is rarely serious and often asymptomatic. This parasite is more an opportunist.
- Oocysts of *Cyclospora cayetanensis* are not stained by iodine.
- Because the oocysts may be passed in small amounts and intermittently, repeated stool examinations and concentration procedures are recommended.
- When freshly passed in stools, the oocyst is not infective (direct faeco-oral transmission cannot occur). In the environment, sporulation occurs after days or weeks (depending on temperature, humidity,…), resulting in an oocyst, containing 2 sporocysts, each containing 2 sporozoites.
- Most oocysts of *Cyclospora cayetanensis* are acid fast. They are variably stained by Ziehl-Neelsen, with different oocysts ranging from colourless to deep purple.
- When stained with the negative staining technique of Heine, the oocysts show as non-stained, strongly refractile structures on a pink to purple background.
- The oocysts can also be detected by their autofluorescence. They light up violet at a wavelenghth of 365 nm, green at 405 nm and blue to violet at 436 nm. This technique can only be used on thin preparations of untreated faeces or faeces treated with SAF or 10% formaline solution (if desired after concentration using sedimentation).

Cryptosporidium spp.

Family : Cryptosporidiidae

Class : Coccidea

Geographic distribution :

Worldwide

Common name :

Disease:

- NL : cryptosporidiose
- FR : cryptosporidiose
- EN : cryptosporidiosis
- ES : cryptosporidiosis

Final host:

- Man
- Various animals

Intermediate host:

Without intermediate host and without vector

Transmission:

Faeco-oral : ingestion of sporulated oocysts.
(Air borne ?)

Localisation of the parasite :

Intracellular, schizogony in epithelial cells of the intestine.
(intracellular, in the lungs, the liver or pancreas: exceptional)

Diagnostic possibilities :

- Oocysts detection in faeces : negative staining technique of Heine, Ziehl-Neelsen staining (both direct or after concentration by sedimentation).
- [Oocysts detection in Bronchoalveolar lavage or in biopsy.]
- Antigen detection in faeces (ELISA, EIA, LFA,…).
- [Serology : antibody detection in serum (IFAT, ELISA, DATS).]
- Detection of specific DNA using PCR.

Morphology of the oocysts :
(in faeces, after specific staining)

Size : 3 -8 µm.

Morphology : Round.

Contents : Small moon-shaped elements (sporozoites)

Characteristics : Oocysts are acid fast (staining pink to red with Ziehl-Neelsen).

Main associated biological signs :

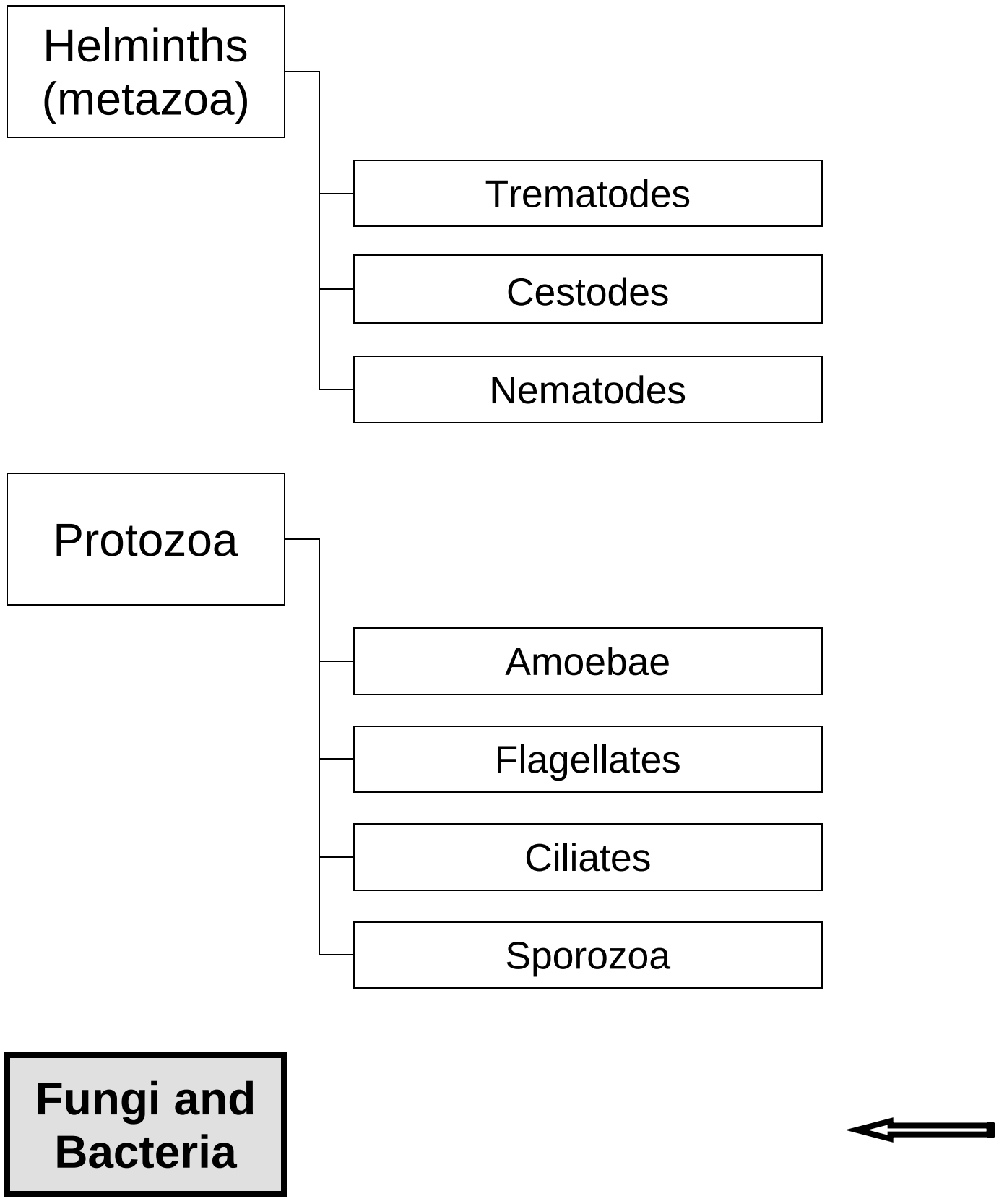
- Watery diarrhoea without blood.
- Often presence of Charcot-Leyden crystals in the faeces.

Possible confusion with :

- *Cyclospora* spp. (Ziehl-Neelsen).
- Yeast (Ziehl-Neelsen).

Remarks :

- Human infection with *Cryptosporidium* spp. is rarely serious. In immunocompetent persons, symptoms are usually short lived (1 to 2 weeks); they can be chronic and more severe in immunocompromised patients. This parasite is more an opportunist.
- The oocyst is directly infective (direct faeco-oral transmission can occur) and remains infective for extended periods. Auto-infection can occur. Great care should be taken in places where immunocompromised patients reside.
- The identification of *Crysptosporidium* oocysts in direct preparation or with iodine staining is impossible. Specific staining is needed.
- *Cryptosporidium* spp. is mainly found in water (drinking or recreational water). Oocysts are resistant to the concentration of chlorine normally used for swimming pools or for the treatment of domestic water supplies.
- *Cryptosporidium parvum* and *C. hominis* (together known as *C. parvum* genotype 1) are the main species infesting man. Infestations caused by *C. felis* (cats), *C. meleagridis* (birds), *C. canis* (dogs), *C. nasorum* (fish), *C. muris* (rodents), … have been reported however. Host specificity does not appear to be strict.
- When stained with the negative staining technique of Heine, the oocysts show as non-stained, strongly refractile structures on a pink to purple background. This staining technique is a good technique for screening samples.
- Most oocysts of *Cryptosporidium* spp. are acid fast. They stain pink to red by Ziehl-Neelsen, some oocysts will not stain at all.


Helminths (metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Pneumocystis jiroveci

Family : Pneumocystidaceae

Fungus

Class : archiascomycetes

Geographic distribution :

Worldwide

Common name :

Disease:

- NL : pneumonie door *Pneumocystis carinii*
- FR : pneumonie à *Pneumocystis carinii*
- EN : *Pneumocystis carinii* pneumonia
- ES : neumonia *Pneumocystis carinii*

Final host:

- Man

Intermediate host:

?

Vector ?

Transmission:

airborne ?

Most healthy children have a positive serology
→ reservoir ?
→ latent infections ?

Localisation of the parasite :

- Lungs
- (Extra-pulmonar dissemination)

Diagnostic possibilities :

- X-ray of the lungs.
- Parasite detection in bronchoalveolar lavage, induced sputum or biopsy : Toluidine blue O, Giemsa staining, RAL 555, …
- Detection of specific DNA using PCR.

Morphology of the parasite :

"Trophozoites" : Or small oval with one nucleus
2 – 4 µm (immature)
Or amoeboid with one nucleus
4 – 10 µm (mature)

"Pre-cysts" : 2 to 6 nucleus, tick shell,
3 – 6 µm

"Cysts" : contain 8 parasites, tick shell,
4 – 8 µm

"empty cysts" : Tick shell,
4 – 8 µm

Main associated biological signs :

- immunodepression.

Possible confusion with :

- *Histoplama capsulatum*.

Remarks :

- Toluidine blue O: The walls of cysts or pre-cyst are stained in blue.
- Giemsa: Trophozoites and internal structures of the cysts are stained in blue with a red nucleus. Often only the nuclei will stain. The cyst walls always remain unstained.
- *Pneumocystis carinii* is the old name for *Pneumocystis jiroveci.*

Borrelia spp.

(Not considered: *B. burgdorferi*)

Family : Spirochataceae

Bacteria

Class : Spirochaetes

Geographic distribution :

Worldwide and epidemic
(louse borne borreliosis)

Regional and endemic
(tick borne borreliosis)

Common name :

Disease:

- NL : Recurrente koorts, Borreliose (teken~ of luizen~)
- FR : Fièvres récurrentes à poux ou à tiques, Borreliose.
- EN : Relapsing fever, louse or tick borne Borreliosis.
- ES : Borreliosis, fiebre recurrente por piojos o por garrapatas.

Final host:

- Man
  (louse borne borreliosis)
- Man and animals
  (Tick borne borreliosis)

Intermediate host:

(Vector)
(louse borne borreliosis)

Pediculus humanus corporis

(Vector and reservoir)
(Tick borne borreliosis)

Ornithodorus spp.

Transmission:

- Crushing an infested vector on the skin of the mucosa. **(louse borne borreliosis)**
- Bacteria injected with saliva through the skin when an infested vector takes a blood meal. **(tick borne borreliosis)**
- (Blood transfusion : rare).
- (Congenital : exceptional).

Localisation of the bacteria :

- Blood (CSF).

Diagnostic possibilities :

- Bacteria detection in blood : direct examination, Giemsa stained tick (or thin) blood film, Woo
- (Bacteria detection in CSF or urine : direct examination, Giemsa staining.)
- [Serology : antibody detection in serum]

Morphology of the bacteria :

Fine and long bacteria,

Helical bacteria

10-20 µm x 0,5µm

Stain mauve-blue (Giemsa stain)

actively motile (direct examination)

Main associated biological signs :

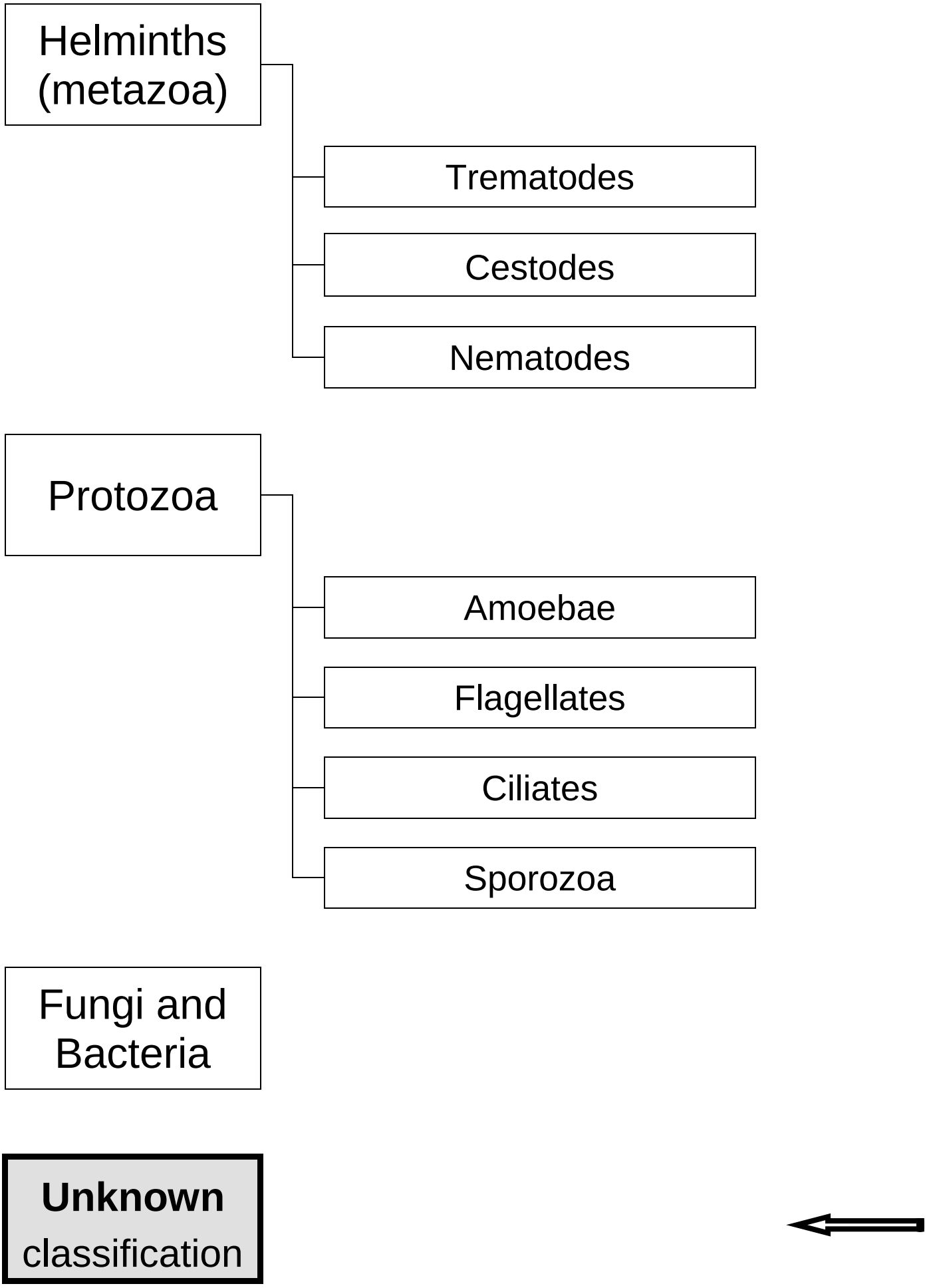
- Fever
- Thrombocytopenia, often perturbation of coagulation tests.
- Often anaemia.
- Leukocytosis (in 30% of the cases) or leukopenia

Possible confusion with :

- *Treponema* spp.
- *Leptospira* spp.
- *(*exflagellation of *Plasmodium* spp. gametocytes)

Remarks :

- The *Borrelia* species which cause relapsing fever cannot be distinguished morphologically. In tick borne borreliosis, fewer bacteria are found in the blood than in louse borne borreliosis.
- The blood should be collected during periods of fever because this is when the bacteria are at the highest concentration.
- Several serological tests have been developed for diagnosing relapsing fever, but antigens for the tests are not generally available and show high cross-reactivity with *Treponema* spp.
- For Lyme disease, caused by *Borrelia burgdorferi* (transmitted by hard ticks, but not causing relapsing fever), blood examination is not recommended (too few bacteria present in blood). Good serological tests are available.
- **Louse borne borreliosis :** *Borrelia recurrentis* : still epidemic in the highlands of Central and East Africa and less frequent in North Africa and the South American Andes.
- **Tick borne borreliosis :** Occur sporadically worldwide **:** *Borrelia duttoni* (Madagascar, Central Africa,…); *Borrelia hispanica* (Central and North Africa, Spain, …); *B. persica* (Egypt, Iran, …); *B. turicatae, B. parkeri* and *B. hermsii* (USA); *B. venezuelensis* (South America);… For tick borne borreliosis, transovarian transmission occurs in the vector.


Helminths (metazoa)
Trematodes
Cestodes
Nematodes
Protozoa
Amoebae
Flagellates
Ciliates
Sporozoa
Fungi and Bacteria
Unknown classification

Blastocystis hominis

Family : Opalinidae

"incertae sedis"

Class : Blastocystea

Geographic distribution :

Worldwide

Common name :

Disease:

NON PATHOGENIC ?

Final host:

- Man
- ?

Intermediate host:

Without intermediate host

Vector ?

Transmission :

Faeco-oral, cysts-like ingestion ?

Localisation of the parasite :

Intestinal lumen.

Diagnostic possibilities :

- Detection in faeces : **Direct examination.**
  concentration by sedimentation (only on samples fixed by formalin).

Morphology of the cysts-like :

Size : 5 - 40 µm.

Morphology : Round or oval.

Contents : Large central vacuole surrounded by small, multiple nuclei**.**

Main associated biological signs :

NON PATHOGENIC ?

Possible confusion with :

- cysts of various protozoans

Remarks :

- Knowledge of the life cycle of *Blastocystis hominis* and transmission is still under investigation. After being classified with the fungi and latyer on with the algae, it is now considered tob e one of the protozoans (Cavalier-Smith, 1998). Its (possible) pathogenic capacities may be linked to the degree of infestation (more than 5 *Blastocystis*-forms per microscopic field in direct examination at 400x) or to the presence of other organisms, together with *Blastocystis*.
- Whether *Blastocystis hominis* can cause symptomatic infection in humans is a point of active debate. This because of common occurrence of the organism in both asymptomatic and symptomatic persons.
- Staining with Lugol's staining solution (according to d'Antoni) makes visualization of this organism easier.
- Concentration techniques will lyse the organisms, except if the specimen has been fixed by formalin.

Armillifer spp.

Family : Armilliferidae

"incertae sedis"
Class : Maxillopoda (subphylum Crustacea) ?

Geographic distribution :

- Africa (*A. armillatus, A. grandis*)
- Asia (*A. moniliformis*)

Common name :

Tongue worm

Disease :

- NL : porocephalose, pentastomiase
- FR : porocéphalose, pentastomiase
- EN : porocephalosis, pentastomiasis
- ES : porocefalosis, pentastomiasis

Final host :

- Snakes

Intermediate host :

- Small (herbivorous) mammals
- Rodents
- (Humans)

Transmission :

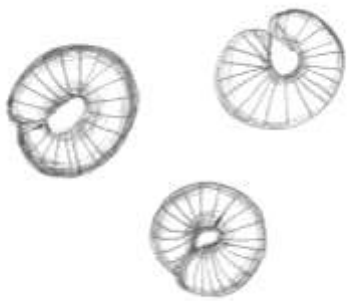
- Consumption of undercooked, infested snake meat.
- Drinking water, contaminated with excretions or secretions of infested snakes.

Localisation of the nymphs:

Liver, spleen, lungs, intestinal wall,…

Diagnostic possibilities :

- Visualisation of the typical larvae or nymphs.
  - Macroscopically, as an accidental finding during surgery or during autopsy.
  - As typical C-shaped calcifications during medical imaging.



Morphology of the nymphs :

Dimensions : 1 to 2 cm long, about 2 mm thick

Aspect : enrolled, cylindrical larvae, usually C-shaped

Characteristics : about 20 to 30 "rings", depending on the species

Associated biological signs :

- Usually asymptomatic
- Mild hypereosinophilia is possible

Possible confusion with :

Remarks :

- *Armillifer* species are at this point considered to be arthropods belonging to the subclass of Pentastomida or "tongue worms". Their classification is however still enigmatic. In the past, they have been considered to be nematodes, annelids, arachnids, crustaceans,…
- The *Armillifer* species most encountered in humans are *A. armillatus, A. moniliformis* and *A. grandis.*
- Other Pentastomids sporadically infesting humans are *Linguatula serrata* and *Porocephalus crotali*.

Diagnostic techniques in parasitology

Parasitological examination of faeces

The examination of faeces (or coprological examination) aims to detect those parasites that leave their host by the digestive tract and are thus found in the stools.
When taking a stool sample great care should be taken not to contaminate the sample with urine (this can cause distortion of vegetative forms of protozoans). Also avoid tests that can interfere in the coprological examination (e.g. after a Ba-transit or enema the patient should wait at least one week before delivering a faecal sample to the lab). The sample should be given to the lab in a proper packing. The faeces should never be packed in absorbing materials (paper, cardboard, matchboxes, etc.) Ideal are plastic or glass air-tight containers. These are however not always accessible in tropical regions. In this case, one can also use a fresh banana-leaf.
When the sample arrives in the laboratory, it should first be checked for its consistency, presence of blood or mucus and presence of worms or parts of worms. The microscopic examination should be done as soon as possible, giving priority to liquid and/or bloody samples, because in these samples one can possibly find living protozoans which will decompose (or at least immobilize) relatively quickly after production of the sample. This will complicate (or even make impossible) their identification. Samples that cannot be examined immediately should be stored at +4°C. When samples are stored for a long period of time, or when a sample should be sent by mail, it is advisable to add a product to the stools that prevents fermentation without altering the aspect of possible parasites (formaldehyde, merthiolate, SAF,…)
Some limitations of the parasitological examination of stools:

- The secretion of parasites or parasitic elements can sometimes be intermittent. When clinically suspecting someone of a parasitic infestation, a second examination of stools, produced a few days later, can prove very useful.
- In the rare case of infestation with only male helminths, no eggs or larvae will be secreted.
- Nor is there any secretion of parasitic elements during latent periods, e.g. maturation of the parasite in the body of its host, periods of internal migration, ...
- Some parasites of the intestinal tract cannot be detected by looking for parasitic elements in the stools of the host (e.g. eggs of *Enterobius vermicularis* which are placed on the peri-anal skin, eggs of certain tapeworms which are secreted in intact segments,…). This compels us to carefully study the biology of these parasites.
- Sometimes, the eggs found in a stool sample do not indicate an actual infestation, but are merely "passed" in the stools by eating contaminated foods (e.g. eggs of Fasciolidae in liver).
- …

Fixation of a stool-sample

This technique (especially intended for detection of trophozoïtes) avoids degeneration of possibly present parasites in a stool-sample, while preserving the parasite's morphology. This allows identification of the parasites after permanent staining (e.g. Iron-Hematoxylin Staining of Kinyoun (see p. 104)).
Mix thoroughly at least 3 volumes of SAF (see p. 124) with 1 volume of stools (as soon after production as possible; max 15-30 minutes). The stools and SAF should be mixed well by shaking for at least 20 seconds. The recipient containing SAFshould have a label indicating the dangers of SAF and the final level of liquid (after the stools have been added).

Direct examination

Direct examination consists of a microscopic examination of a faecal sample, before any other products are added. This examination should be made as soon as possible after receiving the sample at the lab.
When the faecal sample is liquid, one can simply take a drop of faeces and place it on a slide. Cover this drop with a cover slip (20mm x 20mm). This preparation should be examined completely and systematically with the 10x objective (total magnification of 100x), looking for eggs, larvae and ciliata. Details are observed using the 20x or 40x objective. The searching itself is done in any case with the 10x objective. After that about 100 microscopic fields are examined using the 40x objective (total magnification of 400x). At this moment one should look only for cysts and/or vegetative stages of protozoans (possible cysts and/or trophozoites of *Balantidium coli* are found already with the 10x objective).
When the stool sample has a (more or less) normal consistency, a drop of saline is first put on a slide. Then, a small amount of the faecal sample is put into suspension by means of a wooden spatula. The whole is covered with a cover slip (20mm x 20mm). Examine as described above.
The quantity of faeces examined this way is about 2 mg.

Modified Kato-Katz technique

This method uses a rather large amount of faeces (±0,05 g). The faeces is placed on a glass slide. In order to render this thick layer of faeces sufficiently transparent for microscopic examination, it is covered with a piece of cellophane (30mm x 30mm) that has been soaking for at least 24h in glycerinewater (30/70 v/v). The whole is slightly flattened and examined microscopically after ½ to 24h. The clarification-time needed, depends on the egg that is looked for and on the glycerine-concentration used. This technique is only useful in helminthology.
The disadvantage of this technique is that, after some time, all the eggs will become transparent and thus invisible. Thin-shelled eggs will clarify a lot sooner than thick-shelled ones, rendering this technique inappropriate for routine-analysis. However, this technique is very useful when looking for only one kind of egg (e.g. *Schistosoma mansoni* eggs), and when all other eggs that might be present are of no interest. In this case, the incubation-period can be adapted to the egg one is looking for. This technique is widely used for epidemiological purposes. When desired, the result can be expressed in a quantitative way (using a constant amount of faeces).

Lugol staining of wet smears for cysts of protozoa

This technique facilitates identification of cysts of protozoa. The iodine in the Lugol's staining solution will colour the cysts yellow to yellow-brown, colouring the chromatine of the nuclei more intensely than the cytoplasm. This will render the morphological aspects of the nuclei more visible.
A small amount of stools (fresh stools or concentrated material) is put in suspension in a drop of Lugol's staining solution, using a wooden spatula. The Lugol's staining solution (d'Antoni's Lugol-solution) used for parasitology is more concentrated than the one used for bacteriology. When using a staining solution which is not concentrated enough, the cysts won't take up enough colour and their identification will become even harder. The preparation should not be made too thick, as this will cause the cysts' internal structures to become practically invisible. Preparations are examined with a 40x objective.

Negative staining according to Heine

This staining technique is used especially to look for oocysts of *Cryptosporidium* spp. A small drop or amount of faeces (if desired concentrated material can be used) is placed on a glass slide. Add 1 to 3 small drops of carbol-fuchsin solution. Mix well and prepare a (not too thin) smear from this suspension. Let the preparations dry on the bench (the smears should be dry within 15 min, so don't make the smears too thick either). Place a drop of immersion-oil on the preparation and examine with the 100x objective.
The *Cryptosporidium* oocysts will show as small, round, non-stained and highly refractile structures of about 3 to 6 µm in diameter. The background will be pink to purplish.
As the use of the 100x objective drastically lowers the sensitivity, the preparation can be looked at with the 50x immersion-objective (very expensive objective), or the drop of immersion-oil can be covered with a cover slip so the preparation can be examined with the 40x objective.

Modified Ziehl-Neelsen staining

Prepare a thin smear of the faeces-sample (if necessary diluted in saline). Let the preparation dry on the bench and fixate it by placing it in methanol during 2 to 3 minutes. Cover the complete preparation with carbol-fuchsin solution during 10 minutes. Heating isn't necessary when searching *Cryptosporidium* spp.! Rinse in water and destain with acid alcohol untill no colour comes off anymore. Rinse again with water. Counterstain with methyleneblue-solution during 30 seconds. Rinse once more with water and let the preparation dry. Examine the slide with the 50x or 100x objective (oil-immersion).
Cryptosporidium-oocysts will show as small, round, red to violet structures of about 3 to 5 µm in diameter. Internally, some more intensely coloured spots can be observed.
Take care not to confuse the oocysts with yeast-cells!

Iron-Hematoxylin Staining of Kinyoun

This staining technique is very useful to detect infestations with protozoa (and especially trophozoïtes). The picric acid will differentiate the hematoxyline by removing more colour from fecal debris than from protozoa. It also removes more colour from the protozoa's cytoplasm than from their nuclei, resulting in a moderately grey-blue stained protozoa, containing dark-blue to black nuclei, on a rather clear background. Cysts usually stain darker than trophozoïtes. Chromatoïd bodies and phagocytized RBCs stain dark-grey to black. Helminth eggs will stain too intensely and will not be easily identified in the stained preparation. Acid-fast structures will stain clearly pink to deep-purple.
Identify 1 clean glass slide per patient. Mix 1 drop of Mayer's Albumin (see p. 123) with 1 drop of feces, fixed with SAF (see p. 124). Make a thin smear, using a wooden stick (e.g. cotton swab) and let it dry at room-temperature for 20 minutes to 2 hours.

Staining procedure: (place the slides on a paper tissue between each staining step to minimize carry-over!!!)

- fix in ethanol 70% (see p. 120) during 10 minutes
- wash in tap-water (not running) during 2 minutes
- stain in carbol-fuchsin solution (see p. 120) during 20 minutes
- wash in running tap-water during 1 minute (constant stream of water in container)
- destain in hydrochloric alcohol (see p. 120) during 2 minutes
- wash in running tap-water during 1 minute (constant stream of water in container)
- stain in iron-hematoxylin working-solution (see p. 122) during 8 minutes
- wash in demineralised water (not running) during 1 minute
- destain in picric acid working-solutiong (see p. 124) during 4 minutes
- wash in running tap-water during 10 minutes (constant stream of water in container)
- dehydrate during 3 minutes in alcohol-ammonia solution (see p. 120)
- dehydrate during 5 minutes in ethanol 100%
- dehydrate during 5 minutes in a second container with ethanol 100%
- dehydrate during 5 minutes in xylol
- dehydrate during at least 5 minutes in a second container with xylol

Place a few drops of Entellan® onto the wet preparation and cover with a coverslip. Let the preparation dry overnight at room-temperature and examine with a 50x (or 100x) oil-immersion objective during 5 to 10 minutes. Morphological details should be looked at with a 100x oil-immersion objective.

- The iron-hematoxylin working-solution should be checked before each run by adding a few drops of working-solution to a recipient with (alkaline) tap-water. A blue colour should develop.
- The staining containers should be covered after each run to avoid evaporation of reagents.
- The iron-hematoxylin working-solution, the picric acid working-solution, the 70% ethanol fixation-step, the destaining-step with hydrochloric alcohol and the dehydration-step with ammonia-alcohol solution should be replaced each week.
- The washing-steps with tap-water and with demineralised water should be freshly prepared each run.
- The carbol-fuchsin solution should be replaced each month.
- The steps with absolute ethanol and with xylol should be replaced when becoming "foggy" or when visible accumulation of water at the bottom of the container occurs.

Concentration-techniques

The goal of these techniques is to concentrate any possible parasites, present in small numbers, to a smaller volume of faeces. This smaller volume of faeces, having a higher concentration of parasites in it, can then be examined microscopically. These concentration-techniques however will kill any trophozoites of protozoa, present in a sample. This means that concentration-techniques can never replace a direct examination, but can only be used as supplementary techniques.
Many variations exist in this group of techniques, but most of them are based on differences in specific gravity. Some examples of specific gravities:

	specific gravity
Zinc-sulphate 33 % solution in water	1,180
Formol-solution 10 % in saline	1,019
Ether	0,714
Egg of *Ancylostoma duodenale*	1,055
Cyst of *Giardia lamblia*	1,060
Cyst of *Entamoeba histolytica*	1,065 - 1,070
Cyst of *Entamoeba coli*	1,065 - 1,070
Cyst of *Endolimax nana*	1,065 - 1.070
Egg of *Ascaris lumbricoides* (fertilized)	1,110
Egg of *Trichuris trichiura*	1,150
Cyst of *Chilomastix mesnili*	1,180
Egg of *Ascaris lumbricoides* (unfertilized)	1,200

The most simple and widely used techniques will be discussed here.

Sedimentation-technique: technique according to Loughlin & Spitz

This technique suspends faeces in a low-density fluid (saline). Faecal residues are dissolved in ether (defatting agens). After centrifugation, the parasites can be found in the sediment. This technique concentrates all helminth eggs and larvae, as well as all cysts of protozoa.

- suspend about 3g of faeces in about 42ml of saline (formolated if desired) and homogenize well
- transfer about 1,5ml of this suspension to a conical centrifugation-tube
- add about 3,5ml of saline (formolated if desired)
- add 5ml of ether (or gasoline if no ether is available)
- if desired, 2 drops of xylol can be added
- close the tube firmly with a rubber stopper and shake vigorously during about 1 min.
- remove stopper and centrifuge at 2000 rpm (± 650 *x g*) during 3 to 5 minutes

← ether and fatty acids

← coagulum

← (formolated) saline

← sediment (containing parasites if any)

- after centrifugation, 4 layers can be observed
- if necessary, the coagulum is loosened from the inner wall of the centrifugation-tube, using a spatula
- the centrifugation-tube is poored out with one supple movement
- transfer the sediment to a glass slide, using a pipette
- cover with a cover slip and perform a systematic, microscopic examination

CAUTION:

Ether is an extremely flammable and/or explosive substance when brought in contact with an open flame or spark. Recipients, once opened, are to be kept in a high (dispersion of fumes) and cool place in the lab. As ether is very volatile, recipients should be closed well and hermetically. A recipient with ether should NEVER be kept in the refrigerator: ether-fumes will build up and the whole may explode when opening the fridge (spark of refrigerator-light). Recipients in use should never be placed together in a cupboard. Avoid having large amounts of ether in the lab.

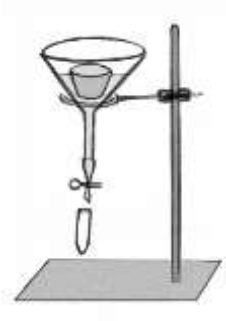
Flottation-technique: modified technique according to Willis

This technique will cause low-density parasitic elements to float on the surface of the flotation-fluid. This method can be used to concentrate cysts and oocysts of protozoa, as well as for eggs of cestodes and nematodes. This concentration-technique however can not be used to concentrate trematode-eggs, because of their high density. Saturated solutions of NaCl or $ZnS0_4$ can be used as flottation-fluids.

- suspend about 5g of faeces in about 100ml of flotation-fluid (homogenize well)
- sieve if desired
- poor the (sieved) suspension in a narrow tube (untill the surface becomes convex)
- gently place a cover slip (20mm x 20mm) on the liquid-surface
- let stand for about ½ hour (parasites will float up against the cover slip)
- transfer the cover slip (in horizontal position) to a glass slide and completely examine microscopically

Baermann-concentration for larvae

This (very sensitive) technique is meant to demonstrate larvae of *Strongyloides stercoralis*. The test is based on hygro- en thermotropism of the larvae. The test can only be done however on well-formed, freshly produced faeces (soft and liquid stools and faeces samples of more than 6h old cannot be used).



About 20g of FRESHLY PRODUCED and WELL-FORMED faeces is placed in a sieve (meshes ±1 mm) (if desired the stools can be placed in the sieve on a piece of gauze, making it easier to remove the faeces afterwards). In turn, the sieve is placed in a glass funnel, to which a piece of rubber tubing is attached. The rubber tubing is closed firmly with a clamp. Carefully fill the funnel with luke-warm water (30-35°C) until the faeces are practically submerged. Let the whole stand for at least 6h (e.g. overnight). Next, collect 10ml of the water in a conical centrifugation-tube by carefully opening the clamp. Centrifuge at 2000 rpm (± 650 *x g*) during 10 minutes. Carefully poor out the tube (immediately after centrifugation) and examine the sediment (WITHOUT adding Lugol's solution) with the 10x objective (presence of (probably living) larvae).

Parasitological examination of urine

Some limitations of the parasitological examination of urine:

- The secretion of parasites or parasitic elements can sometimes be intermittent. When clinically suspecting someone to have a parasitic infestation, a second examination of urine, produced a few days later, can prove very useful.
- In the rare case of infestation with only male helminths, no eggs or larvae will be secreted.
- Nor is there any secretion of parasitic elements during latent periods, e.g. maturation of the parasite in the body of its host, periods of internal migration, ...
- …

For the detection of *S. haematobium* eggs, a sedimentation or a filtration technique can be used. The sedimentation technique is less sensitive than the filtration technique, but is cheaper and is easier to implement in a small laboratory.
The filtration technique is mostly used for epidemiological purposes, when a quantitave result is demanded.

Sedimentation

The urine should be collected in a conical recipient. The sample should be left on the table for about 1 hour, giving it the opportunity to form a sediment. Carefully decant the urine, leaving about 20 ml of the sediment in the recipient. The remaining urine is transferred into two conical centrifugation tubes. Centrifuge at 2500 rpm (± 1000 x *g*) during 5 minutes.
After centrifugation, the tubes should be emptied with one supple movement. Mix the sediment and transfer it to a glass slide. Cover with a cover slip and systematically examine it with the 10x objective (for eggs of *Schistosoma haematobium*). Next, about 200 fields should be examined with the 40x objective (for trophozoites of *Trichomonas vaginalis*).

IMPORTANT:

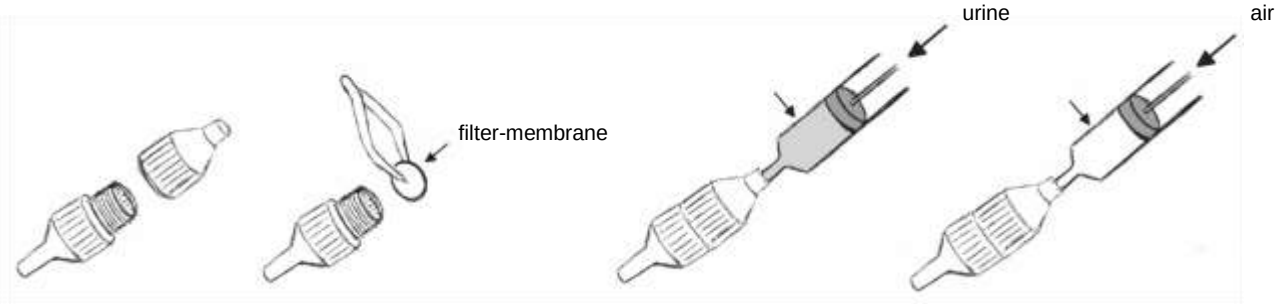
When collecting a specimen for searching eggs of *S. haematobium*, some points should be taken into consideration:

- Collect the urine preferably between 10h00 and 14h00
- Before urinating the patient should move/jump around a little bit
- Before giving a urine-sample, the patient's bladder should be well-filled
- As the eggs are particularly present in the last millilitres, maximum miction is advisable

When searching for *Trichomonas vaginalis* the first fraction of urine should be collected!

Filtration

This is a useful technique to look for eggs of *Schistosoma haematobium* in urine.
The urine sample should be well-mixed. Then 10 ml of urine is pressed through a small filter (meshes of 20 µm). Next, air should be pressed through the filter to clear out the filter.
The eggs will not pass through and thus stay on top of the filter. The filter is transferred to a glass slide and examined with the 10x objective.
Different kinds of filters exist (paper, nylon, polycarbonate). The filter-holders can be opened to place or take out the filters. Except for the paper filters, all filters can be used several times. For this, they should be placed in sodium-hypochlorite-solution (10%) during the night, then rinsed, next placed a few hours in a soap-solution, rinse very thoroughly and dry. The cleaned filters should be checked with a microscope (10x objective) before using them again.
This filtration method allows determination of the number of eggs per ml of urine.



Parasitological examination of vaginal secretions

Using a steril cotton swab, a sample is taken, preferrably from the cervix-mouth. The sample is pressed out immediately on a clean glass slide. The fluid is covered with a cover slip and examined immediately with the 10x and the 40x objectives. If the preparation is too thick, a drop of saline can be added.
This technique can be used to demonstrate infections with *Trichomonas vaginalis.* The parasite will draw the attention by its typical shaky movements, causing any neighbouring cells to move with it. Immobilised parasites will be hard or impossible to identify. This is the reason why samples should be examined immediately (before immobilisation of the parasites).

Parasitological examination of sputum

This test is useful when searching eggs of *Paragonimus* spp*.* in sputum. When examining sputum larvae can sometimes be found of worms, passing the lungs in their cycle of development (e.g. *Ascaris lumbricoides,* Hookworms*, Strongyloides stercoralis,*...). Examination of sputum however is not the best way to search for these helminths!

IMPORTANT: Hemoptesis can be an indication for infestations with *Paragonimus* spp. However, it can also indicate tuberculosis. For this reason, bloody sputum-samples should be checked for acid-fast rods, prior to parasitological examination.

Instructions should be given to each patient on how to produce a good sputum sample. A good procedure on how to collect a sputum sample will improve the final results and lower the risks for health personnel.

The infection risk for health personnel (and other surrounding people) is very real when a patient, suspected of TB, starts coughing. For this reason, the collection of the sputum sample should be done outside, far away from other people, taking into account the direction of the wind. If this is not possible for any reason, a separate, well ventilated room in the building, should be used (verify the position of the patient according to the winds and air-streams coming from opened windows and doors).
Each recipient for the collection of the sputum sample should be labeled in advance (date, name, number, etc.). To avoid sample inversion, these data should be on the recipient itself, rather than on the lid of the recipient. The recipients should be single-use, made of hard plastic, having a lid that screws on and having an opening of about 2 to 3 cm. If this is not possible, recipients of glass (which can be re-used), having the same characteristics, can be used (decontaminate, clean and sterilize before each use of course). The recipients should be made of transparent material to allow visual evaluation of the quality of the sputum sample, without having to open the recipient.

The sputum itself has to be coughed up from as deep in the lungs as possible. This is best achieved by inhaling deeply several times and then exhaling strongly. A volume of sputum of about 3 to 5 ml is preferred and should contain solid or purulent particles (saliva is not a good sample because of the low number of pulmonary bacilla in it and because of the possible presence of commensal mycobacteria. This also counts for nasal secretions). When a patient is being treated for TB, it is possible that the sputum sample is not purulent.
It is best to collect 2 to 3 sputum samples: the first should be collected on the spot (at the first consultation), the second at home (when the patient wakes up in the morning) and if possible a third on the following consultation. The entire processus thus takes two days. Usually the best sample is the early morning sputum. For this reason, some TB programs use two early morning sputums and one sputum on the spot.

A good sputum sample should not look like water. Its colour should be white-yellowish, sometimes with a little bit of blood in it (purely blood is not a good sample). When a sample looks like saliva, it should be refused and the patient should be encouraged to produce a new sample of good quality. If no better sample is obtained (e.g. during treatment), a watery sample can be analyzed however.
The quality of a sputum sample can also be evaluated after preparation onto a slide and after staining: a saliva sample will be very thin, usually containing "bubbles". Under a microscope, a real sputum will contain mucus filaments and WBCs, while a saliva sample will principally contain epithelial cells (a sputum sample has about 20 times more WBCs than epithelial cells).
A sputum sample can be kept for more than one week at room temperature before bacteriological analysis (for acid-fast rods) is done. This however is not the case for cultures and for parasitological analysis.

Direct parasitological examination

After excluding TB, a sputum sample is transferred to a conical centrifugation-tube. Add at least the same amount of 1% NaOH or 1% KOH solution. Close the tube firmly and shake vigourously during about 1 minute. Let it stand for ½ to 1 hour, shake again very vigourously and centrifuge at 2500 rpm (± 1000 *x g*) during 5 minutes.
After centrifugation, the tube should be poured out with one supple movement. Mix the sediment and transfer it to a glass slide. Cover with a cover slip and examine systematically with the 10x objective. The NaOH's or KOH's function is to clear up and liquify the sputum.

Remark: Although several larvae (*Ascaris* spp., Hookworms, etc...) will pass the lungs during their evolution, examination of sputum is not the best technique for diagnosis of these infestations.

RAL 555 staining

This technique is used for the detection of *Pneumocystis jiroveci* in bronchoalveolar lavage or in a lung biopsy or –punction. This technique stains the **inner structures of the cysts**. This technique can be replaced by Giemsa staining (of thin films). Centrifuge the sample at 2000 rpm (± 650 *x g*) during 10 minutes and spread out the sediment on a glass slide, making a thin film (or make prints of the biopsy on a glass slide). Let the preparation dry at room temperature, then fix the slide using methanol. Place the slide during 25 seconds in the RAL-reagent 2 (watery eosin-solution, code RAL 361643). Without rinsing the slide, place the slide for 40 seconds in RAL-reagent 3 (watery methylene-blue-solution, code RAL 361653). After rinsing the slide using running tap water, let it dry at room temperature. Examine the preparations microscopically using 10x eye-pieces and a 100x objective. Information concerning RAL reagents and their distributors can be found at http://www.reactifs-ral.fr (french).

Toluidine blue O staining

(Chalvardjian et al., 1963, modified by Marty et al. 1981)
This technique is used for the detection of *Pneumocystis jiroveci* in bronchoalveolar lavage or in a lung biopsy or –punction. This technique stains the **cyst walls**. Centrifuge the sample at 2000 rpm (± 650 *x g*) during 10 minutes and spread out the sediment on a glass slide, making a thin film (or make prints of the biopsy on a glass slide). Let the preparation dry at room temperature, then fix the slide using methanol. Place the slide during 5 minutes in sulfatation reagent. Rinse the slide using running tap water. Place the slide during 5 minutes in Toluidine blue O solution. Pass the slide trough 3 different recipients with isopropyl alcohol (1 minute in each recipient). Let the preparation dry at room temperature and examine microscopically using 10x eye-pieces and a 100x objective.

Parasitological examination of duodenal fluid

This technique is useful for searching eggs of Fasciolidae, Opisthorchidae and *Heterophyes heterophyes* and for searching trophozoites of *Giardia lamblia*.
Duodenal fluid is obtained by tubation. The obtained fluid is centrifuged in a conical test-tube at 2500 rpm (± 1000 *x g*) during 5 minutes. After centrifugation, the tube is poured out. Mix the sediment and examine it with the 10x objective. For *Giardia lamblia* the diagnosis should be confirmed with the 40x objective, if desired, Lugol's staining solution can be added.
Attention: staining with Lugol's solution will kill the trophozoites and identification will have to be done based on morphology!

Tape test

Used for searching eggs of *Enterobius vermicularis*, this test reveals the eggs that are placed on the peri-anal skin by the female adult worms (especially during the evening and night). The procedure is hence best performed early in the morning, before making one's toilet and before defecation.
A trasparent adhesive tape is placed, sticky side out, over the bottom of a round-bottomed test-tube. The tape is then pressed against the peri-anal skin. The eggs, if present, will stick to the tape. Next, the tape is placed on a glass slide and examined with the 10x objective. The tape itself serves as a cover slip. If desired, a drop of saline can be placed between the tape and the slide, to obtain a better image. The ends of the tape should be kept dry (for adhesion).
Remark: Accidentally, eggs of *Taenia* spp. can be found in a tape test (usually it will be the eggs of *T. saginata*).

Examination of segments of *Taenia* spp.

For the differentiation of both *Taenia* spp. the segments can be examined for the number of uterus-ramifications. To render the segments more transparent, they can be cleared up in a 50% glycerine solution for 30 minutes or heated to 60°C in a 5% solution of acetic acid (also ordinary vinegar (as found in any kitchen) can be used without diluting it) for about ½ to 1 hour. Next, the treated segments are pressed between two glass slides and examined in front of a light source. As the segments have become more transparent, the uterus-ramifications can easily be observed and counted.
CAUTION: When manipulating segments or adult tapeworms, always proceed with lots of caution (risk of cysticercosis in case of *T. solium*). Manipulation of segments or adult tapeworms is best done using tweezers. ALWAYS wear protective gloves when manipulating segments or adult tapeworms.

Parasitological blood examination

These techniques are applicable on the following infectious agents: blood microfilaria, *Plasmodium* spp., *Trypanosoma* spp., *Babesia* spp. and some *Borrelia* spp.. Yet each of the following techniques is not always useful for all of these parasites.

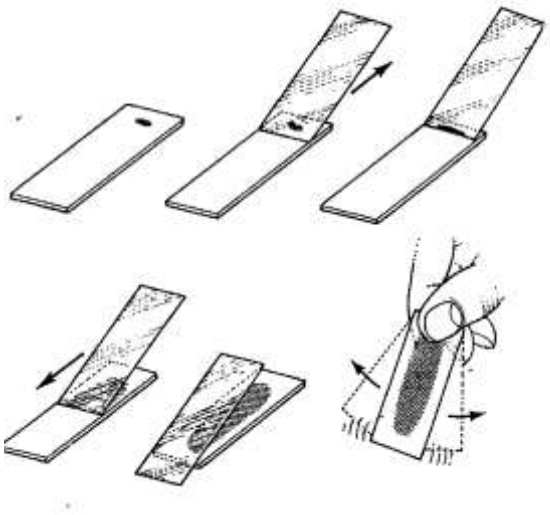
Direct examination

This is the easiest method, but also the least sensible to detect rather big and motile parasites in the blood (trypanosomes and microfilariae). The microscopical examination demands a certain time of attentive observation. An important inconvenience is that the slides must be examined immediately after their preparation (max 10-15 minutes after the bloodsample is taken), especially for searching trypanosomes, since these parasites are recognised by their motility. Nevertheless this test can be of interest, especially on the parasitical peak of *Trypanosoma rhodesiense.* At this moment rather important numbers of trypanosome parasites can be found in the periferal blood, making more complicated and expensive techniques unnecessary. For microfilarae, species identification is not possible. The detection threshold for direct examination is about 15 to 40 microfilaria and 10.000 trypanosomes per ml of blood.

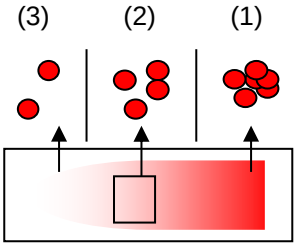
A drop of blood (capillary or venous blood sample taken on anticoagulant, such as EDTA for searching microfilaria or heparin for trypanosomes) is deposed on a slide and covered with a cover slip. The preparation is immediately microscopically examined with objective 10x (or 20x or 40x).

Thin blood film

This low sensitive method in parasitology is only useful for the precise identification of *Plasmodium* species.



A small drop of blood (capillary blood sample or venous blood taken on anticoagulant, such as EDTA for searching microfilaria or heparin for trypanosomes) is placed at one side of a glass slide. Then the small side of another slide (preferably with a polished border) is placed on the first, close to the drop of blood, forming an angle of 45°. Contact of the slide is made with the blood in order to make it spread between the two slides. Then move the upper slide towards the other side. The hand is moved in a steady and uninterrupted way, taking along and spreading the complete drop of blood into a thin and homogeneous layer. Immediately afterwards the slide is shaken between two fingers to dry it instantly in order to avoid deformation of the red blood cells.
A good thin blood film should be thick enough at the starting point (1) becoming less thick in the middle (2) to end in a "flame"-like shape at the end (3). The middle zone is best examined microscopically as the different cells will be lying next to one another here.



The preparation is fixed by covering it with concentrated methanol for 3 minutes.
After evaporation of the methanol, the preparation can be stained using **Giemsa**:
Cover the complete preparation with a 3.5 % diluted Giemsa buffered solution, pH 7.2 (or pH 8.0). For each slide 5 drops of concentrated Giemsa (VWR nr. 1.09204) are added to 4 ml of buffered water in a cylinder. Cover the slides with this solution and stain for 20 to 30 minutes.
Rinse by gently dripping tap water on the preparation. Let dry and examine under the microscope with an oil-immersion objective 100x (or oil-immersion objective 50x).
To obtain a good coloration it is necessary that the diluted water has the correspondent pH for its purpose (see below). Water that is too acid will result in too reddish coloration, while a too alkaline buffer will show a dominating blue coloration.

pH	6.6	6.8	7.0	7.2	7.4	7.6	7.8	8.0
Use	**Hematology**	**Hematology and Leishmaniosis**	**Paludism**	**Paludism**		**Trypanosomiasis**		**Paludism* and Microfilaria**

* pH 8.0 will show possible Maurer's clefts of *P. falciparum*

In the context of a small laboratory it is however preferable to standardise the pH of the dilution buffer to pH 7.2 for all the examinations, including haematological preparations.

To avoid degradation of the Giemsa stocksolution (acid-alkaline reaction), the staining solution should be prepared just before use. The Giemsa stocksolution should also be filtered before use (e.g. when part of it is put in a dripping flask for daily use).

GIEMSA
(hemolysis and staining)

Eosin (acid) ➔ stains the alkaline elements.
Methylene blue (alkaline) ➔ stains the acid elements.
Azur (alkaline) ➔ stains the acid elements.

➔ Fresh dilution of the stain.

➔ Influence of the pH.

The **Pappenheim panoptical staining** (May-Grünwald-Giemsa) can be used instead of the Giemsa staining, but it won't necessarily give a remarkable quality gain:
Once the thin film is dry, it should be covered completely with May-Grünwald solution (VWR nr. 1.01424). Let the solution act for 3 minutes. For this staining technique, fixation of the preparation using methanol is not necessary: the first staining step (using May-Grünwald) causes fixation at the same time as the staining itself. Without removing the May-Grünwald solution, add (drop by drop) 4 ml of buffered water (pH adapted to the intended use: see table above). Let the preparation stand for 1 minute, then remove the staining solution by holding the slide inclined. Without rinsing the slide, cover the complete preparation with a 3.5 % diluted Giemsa buffered solution (pH adapted to the intended use: see table above). For each slide 5 drops of concentrated Giemsa (VWR nr. 1.09204) are added to 4 ml of buffered water in a cylinder. Let stain for 20 to 30 minutes. Rinse by gently dripping tap water on the preparation. Let dry and examine under the microscope with an oil-immersion objective 100x (or oil-immersion objective 50x).

Note : Counting parasites in thin blood films (Malaria and *Babesia spp.)* :

Select an area of the thin blood film where the total number of red cells is approximately 200 per field. Count the number of infected red blood cells in at least 25 fields using the 100x objective and 10x ocular. If red blood cells count in blood is available and accurate (this needs an electronic cell analyser), calculate the number of parasites per µl of blood as follows:

$$\frac{\text{number of parasitized RBC}}{\text{number of examined RBC}} \times \text{RBC-count} = \text{number of parasites / µl of blood}$$

Otherwise, calculate the percentage of parasitized red blood cells as follows :

$$\frac{\text{number of parasitized RBC}}{\text{number of examined RBC}} \times 100 = \% \text{ of infected RBC}$$

The percentage of infected red blood cells can help to monitor patients with heavy parasitaemia.

If the number of RBC/µl of blood is not known, it is estimated that 1% of RBC parasitized corresponds to about 50.000 parasites/µl of blood.

Thick blood film

This method is a concentration technique. It takes about 4 times the blood quantity (20 µl) of the thin blood film (5 µl). Since the blood is less spread out, the eventual parasites are more frequent per surface unit.
It is mainly used for the search for plasmodia, trypanosomes, microfilaria and *Borrelia* spp. but also for the detection of Toxoplasmosis, *Babesia* spp. and others. The detection threshold is in the range of 5 to 10 (100) plasmodia per µl of blood, 1000 trypanosomes per ml of blood and 15 to 40 microfilariae per ml of blood.
A blood drop is placed on a glass slide and immediately defibrinated by mixing with the corner of another slide in circular movements to obtain a blood layer of the correct thickness on a surface of about 1.5 cm diameter. (Defibrination is better than the use of an anticoagulant. If an anticoagulant should be used, EDTA is the best one, except for searching trypanosomes where heparin is preferred) The thickness of the preparation can be taken as correct if a normal print (such as this) can still be seen, but not read, through the film when it is held above the text.

Let it dry without warming up to avoid fixation of the red blood cells, since this fixation will prevent their destruction. The thick blood film should only be stained after complete drying.
Cover the complete preparation with a 3.5 % diluted Giemsa buffered solution, pH 7.2 (or pH 8.0). For each slide 5 drops of concentrated Giemsa (VWR nr. 1.09204) are added to 4 ml of buffered water in a cylinder. Cover the slides with this solution and let stain for 20 to 30 minutes.
[For the detection and identification of microfilaria, the staining time is best doubled to 40 minutes, the staining solution should be two times more concentrated (10 drops of concentrated Giemsa per 4 ml of buffered water) and the staining is best performed at pH 8.0]
The watery Giemsa solution has a double action: haemolysis and staining.
Rinse by ***gently*** dripping tap water on the preparation. Let dry and examine under the microscope with an oil-immersion objective 100x (or oil-immersion objective 50x). The red blood cells will be destroyed by haemolysis, leaving only the white blood cells and eventual parasites behind.
To obtain stainings of good quality the same measurements for the pH of the staining solutions are taken into account (see thin blood film).

Note: Parasite count in a thick blood film (only applicable in the context of malaria):

The search for parasites is considered negative if no parasites have been found during examination of at least 200 microscopical fields with objective 100x (or objective 50x). This takes about 5 to 10 minutes for a well trained technician.

For the parasite count in a thick blood film, the number of asexual parasites forms is expressed per 200 leucocytes (Count as much as necessary parasites in the microscopical fields as to obtain 200 leucocytes). For *Plasmodium falciparum* the gametocytes must be reported separately. Starting from the leukocyte concentration, the number of parasites can be calculated per µl of blood:

$$\frac{\text{Number of asexual parasites counted}}{\text{Number of leukocytes counted}} \times \text{Leukocytes concentration} = \text{Number of Parasites / µl blood}$$

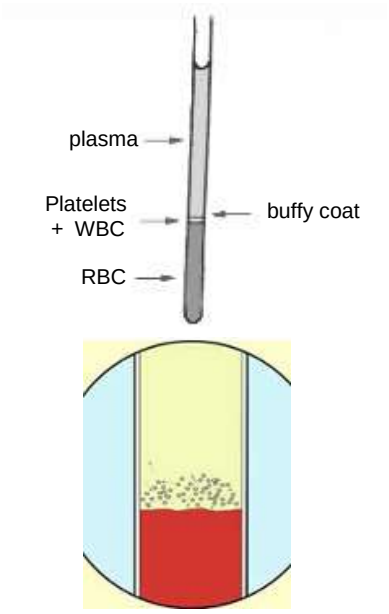
If the leukocyte count is not done, the WHO recommends applying 8.000 leukocytes per µl as a value for all the patients.

In non-immune persons even low parasitaemia can cause pathological phenomena. Especially in non-immune subjects, there is a remarkable correlation between the *P. falciparum* parasite concentration and the seriousness of the symptoms.
(Please note: *P. falciparum* infections examination of series of preparations made each 6 to 12 hours show important fluctuations of parasite concentration. This is caused by the restraint of the parasitised erythrocytes at the moment of schizogony. In infections with synchronical development of parasites, they can disappear temporary from the samples.)

For the routine of a small tropical laboratory, an estimation of the parasitical load in the thick blood film is sufficient for *Plasmodium* spp. This system indicates the relative majority of the asexual parasite forms with a code from 1 to 4 plus, as follows:

1 - 9 asexual parasites / 100 fields =	**+**
1 - 9 asexual parasites / 10 fields =	**++**
1 - 9 asexual parasites / 1 field =	**+++**
> 10 asexual parasites / 1 field =	**++++**

Buffy-coat (WOO)



This method is a rapid concentration technique for detecting motile parasites in blood (trypanosomes or microfilaria). It's easy to perform, but needs an electrical micro-haematocrite centrifuge. This technique is quite sensitive (parasite concentration from 50-60 µl of blood, detection threshold in the range of 250 trypanosomes or 25 microfilaria per ml of blood).

A haematocrite tube filled with 50-60 µl of blood is centrifuged at 12.000 g for 5 minutes. The components of blood separate according to their densities. Trypanosomes and microfilariae will be concentrated above the buffy coat. After centrifugation, the haematocrite tube is placed on a tubeholder and the plasma, just above the buffy coat layer is microscopically examined (10x or 20x objective; 10x or 15x eyepiece). The trypanosomes are very small but can be detected by careful focusing and providing not too intensive light. The preparation must be examined within a few minutes after blood taking and centrifuged, otherwise the trypanosomes will become less active and therefore more difficult to detect. The trypanosomes will also migrate into the supernatant plasma and be missed.
Virtually, all the parasites found in the 60 µl can be visualised by rotating the tube under the microscope.
For microfilariae, the species identification is not possible with this technique. Motile microfilariae will make it impossible to see whether there are also trypanosomes. Breaking the capillaries to obtain a buffy coat for staining with Giemsa is not recommended for safety reasons, but can be used in this case for parasites identification and/or species differentiation.

QBC

The QBC (Qualitative Buffy Coat) test developed by Becton and Dickenson is an expensive concentration method for detecting malaria parasites, trypanosomes or microfilariae in the peripheral blood. The detection threshold is in the range of 100 plasmodia per µl of blood, 250 trypanosomes or 25 microfilaria per ml of blood.

In the QBC system, parasites are concentrated by centrifuging blood in a special capillary tube. This special tube is coated with acridine orange and an anticoagulant. DNA and RNA take up acridine orange stain. The combination DNA-acridine orange fluoresces green after UV excitation and the combination RNA-acridine orange fluoresces green-yellow. Expansion of the centrifugally separated cell layers is achieved with a plastic float. Following centrifugation, the white cells, platelets, and the upper layer of the RBC can be found in the space between the float and inside the wall of the capillary. Examination under a fluorescence microscope (or a special Paralens objective, 50 x), of the interface region between the light RBC and granulocytes, permits detection of malaria parasites (RBC containing plasmodia are less dense than normal ones and concentrate just below the leukocytes).

Malaria parasites fluoresce green-yellow against a dark red background with the nucleus of trophozoites or merozoites of schizontes fluorescing bright green. Trypanosomes and microfilariae will be concentrated above the buffy coat. Motile microfilariae will make it impossible to see whether there are also trypanosomes. Breaking the capillaries to obtain a “buffy coat” for staining with Giemsa is not recommended for safety reasons, but can be used in this case for parasites identification and/or species differentiation.

The equipment, required for the QBC system and the special disposable capillary, are very expensive. Field evaluations of the QBC have shown, to be less sensitive than thick blood films in detecting low parasitaemias (< 100 malaria parasites/µl). Malaria species differentiation is difficult (or impossible) and considerable skill and experience are required to process and examine the tubes correctly and confidently. The use of this technique is not recommended for microfilariae detection, but it’s a good technique for trypanosomes detection.

Knott

Good (concentration-)technique for searching microfilaria at low parasitaemias.
2 ml of blood on anticoagulans is taken by venous punction. The blood is added to 30 ml of 2% formaline solution. This mixture is transferred to 4 conical centrifugation tubes and centrifuged at 1000 rpm (± 160 *x g*) during 15 minutes (or let stand on the bench overnight). The supernatans is decanted and the sediment can either be examined immediately (without the possibility of differentiation of species) or 4 thin smears can be made, left to dry, fixed with methanol during 3 minutes and microscopically examined after Giemsa-staining.

Strout

This technique is used for diagnosis of *Trypanosoma cruzi* infestations.
20 ml of blood (without any anticoagulans) is used. After formation of the blood-clot, the clot is removed and the remaining serum is centrifuged at 2500 rpm (± 1000 *x g*) during 5 minutes. The sediment is examined as is (the parasites will have their motility reduced or even lost, moreover it will be impossible to differentiate between *T. cruzi* and *T. rangeli*) or after Giemsa-staining.

mAECT

This specific technique for trypanosomes detection can be used in research programs or in big screening programs. mAECT (mini Anion Exchange Centrifugation Technique) consists of separating the trypanosomes from venous blood by anion exchange chromatography and concentrating them in the bottom of a sealed glass tube by low speed centrifugation (3000 RPM).
After centrifugation, the tip of the glass tube is examined under the microscope (objective 10x), for the presence of motile trypanosomes. The large blood volume used (300 µl) allows detection of less than 100 trypanosomes/ml. This very sensitive technique requires preparation of the columns (which is a very complex bussiness). Moreover, all reagents have to remain sterile as motile micro-organisms, especially when clumping together, may easily be confused with trypanosomes.
The columns can be ordered for ± 3 $ (in 2005) at:
Prof. J.-J. Muyembe
Institut National de Recherche Biomédicale (INRB)
Avenue de la Démocratie. Kinshasa, R.D. Congo.
E-mail : inrb.rdc@ic.cd.

Parasitological examination of tissues

Concentration technique for the detection of Trichinella larvae

The muscular biopsy is placed into a sieve (meshes of ±1mm), which in turn is placed into a glass funnel, to which a piece of rubber tubing is attached. The rubber tubing is closed firmly with a clamp. Carefully fill the funnel with a digestion-solution of 2% pepsine and 0,5% HCl (see p. 121). Let the whole stand for about 2 hours at 37°C (digestion of the biopsy). Release the clamp carefully to collect the first few drops on a glass slide, cover with a coverslip and examine at low magnification (10x objective – details studied with 40x objective).

Skin tissues

Skin-snip

Good technique for *Onchocerca volvulus* (and *Mansonella streptocerca*).
The skin-snip should be performed close to any onchocercomes, if present, except when the onchocercomes are located in a troublesome area (e.g. the face). Desinfect the target skin-area. The skin is punctured superficially with a sterile needle and pulled upwards a little bit. Next, a thin slice is taken of with a bistouri and placed on a glass slide in a drop of saline. Cover the whole with a cover slip. Examine the preparation with a 10x objective, looking for microfilaria swarming out (½ to 1h after sampling).
This method is useful for screening a large population. Every biopsy is put seperately in a small conical centrifugation-tube, together with a little bit of saline. After about ½ to 1h the biopsy is taken out and the remaining fluid is centrifuged at 2500 rpm (± 1000 *x g*) during 5 minutes. The tubes are gently poured out and the sediment is examined on a slide with cover slip, without coloration (without possibility of differentiation of species) or after Giemsa-staining.

Deep scarification

Good technique for *Onchocerca volvulus* (and *Mansonella streptocerca*).
The scarification should be performed on the same skin-areas as the skin-snip (see above). After thoroughly desinfecting the skin, it is etched with the tip of a bistouri or small needle over a length of about 1 to 1½ cm. The skin should be torn more or less, liberating the lymphatic fluid. While doing this, enough pressure should be applied around the incision, causing a small amount of blood to appear. Three incisions should be made in this manner, with a few mm distance each. Next, enough pressure should be applied around the incisions with index and thumb, causing some blood and lymphatic fluid to surface. Collect a mixture of lymphatic fluid and blood by applying a glass slide to the incision-area. Apply as much material on the glass slide as possible. Let dry and stain with Giemsa.
Using this method, not only skin- but also blood-microfilaria, *Trypanosoma* spp., *Plasmodium* spp. and *Borrelia* spp. may be found. It is evident that scarification is not the appropriate technique for diagnosing these parasites.

Skin-biopsy for Leishmania spp.

The biopsy is to be taken at the outer edge of the leasion. Using sterile tweezers, prints of the biopsy are made onto clean glass slides. Cell-clusters will remain on the glass slide (=“tissue-clash”). Let the preparation dry, fix with methanol and stain as if it were a thin film (see p. 110).

Exsudate

The exsudate on superficial skin- or mucosal leasions can be used to search for *Leishmania* spp. (cutaneous and mucocutaneous leishmaniasis).
Using a pipet or sterile needle a drop of the exsudate is transferred to a glass slide and spread out a bit. Let the preparation dry, fix with methanol and stain as if it were a thin film (see p. 110).

Bone-marrow

Can be spread out as a thin film (see p. 110) or as soft tissue (zie below: “Splenic and hepatic biopsy”). Let the preparation dry, fix with methanol and stain as if it were a thin film (see p. 110).

Splenic and hepatic biopsy

Soft tissue can carefully be spread out onto a glass slide (= “tissue-film”). Let the preparation dry, fix with methanol and stain as if it were a thin film (see p. 110).
For more resistant tissues, prints can be made onto a glass slide, using sterile tweezers. Cell-clusters will remain on the glass slide (=“tissue-clash”). Let the preparation dry, fix with methanol and stain as if it were a thin film (see p. 110).

Rectal biopsy

The biopsy is flattened between two glass slides, without staining or fixation, and examined with the 10x objective (for eggs of *Schistosoma* spp. or trophozoites of *Balantidium coli*) or with a 40x objective (for trophozoites of *Entamoeba histolytica*).

Parasitological examination of lymph node aspirate

The standard method of diagnosis of African trypanosomiasis (especially in gambiense infections) in the early stage is to search for trypanosomes in aspirates from enlarged cervical lymph nodes (adenopathy). Trypanosomes will first invade the lymph nodes (possibly even before antibodies are formed). The swollen lymph nodes usually are those at the base of the patient’s neck. They usually will be round, elastic and can be moved around under the skin. Most of the time these lymph nodes will not be hard, not resistant to pressure and will not be painful. The presence of such swollen lymph nodes is a very suspicious sign in endemic regions, but will however be absent in 20 to 40% of positive cases. Also the contrary can be true: in several cases the lymphatic fluid will be positive for Trypanosomes, while the blood examination remains negative. For this reason, it is absolutely necessary to do a systematic palpation of the cervical lymph nodes during a survey and to aspirate all suspect lymph nodes.

Immobilize the lymph node between thumb and index and carefully insert a needle in the centre of the lymph node. Turn the needle a bit, while massaging the lymph node. remove the needle from the lymph node and carefully place it onto a syringe with the piston pulled to the end. Place a drop of aspiration onto a glass slide by slowly pressing the piston inward. Examined immediately as a wet preparation under a microscope (40x objective). The wet preparation must be examined as soon as possible after the fluid has been collected. This because trypanosomes are unable to survive for more than 15-20 minutes in an aspirate once it has been taken. The organisms become rapidely inactive and will be lysed.

The contents of the needle may also be used to prepare a thin film. After fixation in methanol and staining by the Giemsa method, examine the preparation under the microscope with an immersion objective 100x (or objective 50x).
This procedure is only recommended for *Toxoplasma* detection or amastigotes forms of *Trypanosoma cruzi* or *Leishmania* spp.

Parasitological examination of cerebrospinal fluid

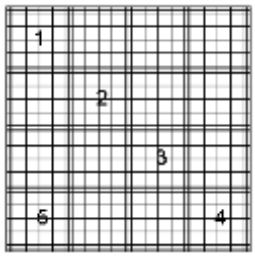
This technique is used for stage determination, follow-up, and sometimes also (in case of clinical suspicion) for diagnosis of *Trypanosoma gambiense or rhodesiense* infestations. Of course, the same technique may be used for *Toxoplasma*, *Cryptococcus*, *Naegleria*, *Acanthamoeba* detection in CSF and also for eosinophlic meningitis (*Angiostrongylus*) or bacterial meningitis.

After collecting the CSF sample, the analyse should be carried out as soon as possible since cells and parasites start to lyse rapidly (10 minutes for trypanosomes for example).
For trypanosomiasis, stage determination is necessary to choose an appropriate treatment with minimal risk for the patient. The second meningo-encephalitic stage is caused by trypanosome invasion in the central nervous system. The only way to determine whether the trypanosomes have invaded the central nervous system is by examination of the CSF, assuming that the changes observed in the CSF reflect the events going on in the central nervous system. Different parameters may be used : white blood cells number, total protein or globulin concentration, presence of trypanosomes, …).
The definition of normal CSF is based on a cell number ≤ 5 cells/µl, the absence of parasites, and a low level of protein concentration. Since there is no close relationship between these parameters, the WHO recommends the use all of them together.

Leukocyte count

The same technique is also used in haematology and in bacteriology (cf. haematology and bacteriology notes). Due to the low number of cells in normal CSF, cell counting chambers used for CSF counting should be medium or big volume counting chambers (Fuchs Rosenthal, Malassez, Neubauer, …). It may be good to use disposable KOVA counting chambers (less volume error, but for about 0,1 € per test). Carefully clean and mount the cell counting chamber (absence of dust or oil, use of special cover slip, presence of Newton rings). Gently mix the fresh and un centrifuged CSF before filling the chamber. If the liquid overflows into channel, the procedure should be restarted. Leave the counting chamber for 5 minutes on the bench to allow the cells to settle. Count the cells with the 10 x objective as explained below (depending on the type of counting chamber). If necessary, use the 40 x objective to make sure that the cells you count are leukocytes and not red blood cells. It is a good habit to do a second counting when the result is less than 20 leukocytes/µl.

Fuchs Rosenthal counting chamber



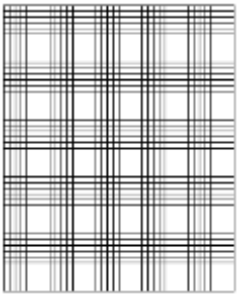
Area : 4 mm x 4 mm

Depth : 0.2 mm

Total volume: 3,2 mm³ or µl

For an undiluted CSF, count the cells in squares 1, 2, 3, 4 and 5. The number of cells counted in these 5 squares equals the number of cells per µl of CSF.

Mallassez counting chamber



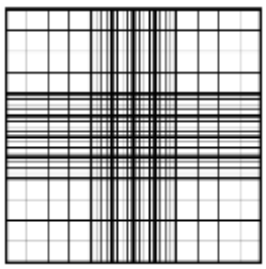
Area : 2 mm x 2,5 mm

Depth : 0.2 mm

Total volume : 1 mm³ or µl

For an undiluted CSF, the number of cells counted in the entire counting chamber equals the number of cells per µl of CSF.

Neubauer (Double Improved) counting chamber



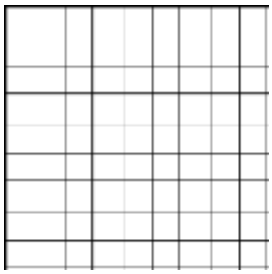
Area : 3 mm x 3 mm

Depth : 0.1 mm

Total volume : 0.9 mm³ or µl

For an undiluted CSF, the number of cells counted in the entire counting chamber multiplied by 10/9 equals the number of cells per µl of CSF.

Kova disposable counting chamber



1 plastic slide contains 10 counting chambers. 1 chamber consists of 9 big squares divided in 81 small squares.

Total Volume : 1µl

Volume of 1 small square : 0,0123 µl

For an undiluted CSF, the number of cells counted in the entire counting chamber equals the number of cells per µl of CSF.

Direct examination

Sometimes, during the cell counting procedure, motile parasites may be detected. It can be trypanosomes, indicating a trypanosomiasis stage II, or amoeboid trophozoites (*Naegleria* or *Acanthamoeba* spp., confusion possible with motile macrophage). Exceptionally, cysts of *Acanthamoeba* spp. may be detected.

Examination after single centrifugation

Several ml of CSF are centrifuged in a conical tube at low speed (10 minutes, 3.000 rpm or 1.000 *x g*) and the supernatant is carefully removed without touching the bottom of the tube. The supernatant may be used for biochemical investigations (protein, glucose, …). The remaining drop or sediment (invisible at low cell counts) is resuspended and a small drop of the remaining fluid is transferred on a clean microscopic slide and covered with a cover slip. The preparation is immediately microscopically examined with a 10 x objective (oculars of 10 x or 15 times) to confirm presence of trypanosomes or other pathogens. The preparation should be scanned completely and systematically. From the sediment, additional smears for detection of other pathogens can be prepared, which can be coloured with Gram, methylene blue, Giemsa or india ink (cf. bacteriology).

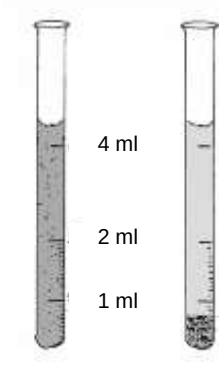
Examination after double centrifugation

The sensitivity of trypanosomes detection in CSF can be improved further by double centrifugation. Several ml of CSF are centrifuged in a conical tube at low speed (10 minutes, 3.000 rpm or 1.000 *x g*) and the supernatant is carefully removed without touching the bottom of the tube. The supernatant may be used for biochemical investigations (protein, glucose, …). The remaining drop or sediment is taken up in one or two micro-haematocrite tubes which are flame-sealed and centrifuged for 5 minutes at high speed (12.000 rpm or 15.000 *x g*). Capillaries are put on a microscopic slide and the cover slip is placed on the sealed end. Clean water is added between the slide and the cover slip to avoid diffraction during microscopic examination (10 x objective, ocular 10 x or 15 x). Proceed as soon as possible : chances of detecting trypanosomes decrease strongly in function of time. [cf. Woo].

CSF total protein

A wide variety in protein quantification methods for CSF is available. These techniques are not well standardised : Different proteins produce different absorbances and the protein concentration values are relative rather than absolute. The different reaction of CSF albumin and IgG with the different reagents used for protein determinations is no exception to this rule [the protein in CSF is mostly constituted of albumin (in normal conditions 70 %) and IgG (30%), which are both derived from the plasma]. Different concentrations may be obtained, depending on the protein used as calibrator (generally pure albumin or IgG). Therefore, various normal reference values for total protein concentration in CSF can be found.
For each method and for each calibrator, the cut-off value has to be established on the spot. Reference ranges vary a lot. The table below is given as example.

Method	Cut off (in g/l)
TCA 5 % precipitation	0,25
Sulfosalicylic acid 3 % precipitation	0,45
Coomassie brillant blue Colorimetry	0,37
BCA method	No data available for CSF



The **Sicard and Cantaloube** method is an easy technique, based on protein coagulation and sedimentation by acid and heat : The Sicard and Cantaloube tube is filled up to 4 ml with centrifuged CSF. The tube is slightly heated to a temperature of 50°C. 12 drop of 30 % (w/v) TCA are added. Close the tube and turn it 3 times to mix the contents (**never shake**). Place the tube perfectly vertically and leave it for at least 5 hours and maximum 24 hours. The height of the sediment indicates the protein concentration. Normal CSF total protein concentrations obtained with this method are below 0,4 g/l

Pandy's globulin test

☠ **CAUTION :** Phenol is a highly corrosive and harmfull chemical. This product must be manipulated with extreme care.

This easy technique may be used to screen for increases in CSF globulin when it is not possible to measure CSF total protein.

Because of it cut off value, this test, based on globulin precipitation by phenol, should be used only on CSF. Pipette about 1ml of saturated phenol solution into a small tube. Place the tube in front of a black background. Using another pipette, add (drop by drop) 3 drops of CSF. Examine the solution after the addition of each drop. If globulins are present, a white cloud forms as the drop of CSF mixes with the reagent (cigarette smoke like). The test is negative if no white cloud forms as the drops of CSF mix with the reagent, or when there is a slight cloudiness that redissolves. A positive Pandy's test occurs in all forms of meningitis, in amoebic and trypanosomiasis meningoencephalitis (stage II), but also in cerebral malaria, brain tumours, cerebral injury, spinal cord compression,…

Formol gel test

This cheap test is a good non specific indicator for the increased serum levels of gammaglobulin. Because of its simplicity, this test may be quite useful to assist in the diagnosis of visceral leishmaniasis (or as screening test for blood-donation?). It is a non specific screening test which if positive requires further investigation. The test is positive in about 90 % of patients with visceral leihsmaniasis. It becomes positive about 3 months after infection and negative about 6 months after a sucessful treatment. Results may be less reliable in case of HIV co-infection. Increased gamma globulin concentrations in serum are also seen following hepatitis B infection, certain malignant diseases (myeloma, Waldnström macroglobulinaemia,…). Increased gamma globulin levels which occur in case of malaria, trypanosomiasis, leprosis,… are not high enough to provided a positive result.

Collect 2-5 ml of blood and allow it to clot. When the clot begins to retract (30 to 60 minutes after collection), centrifuge (10 minutes at 4000 *x g*) the blood to obtain clear serum. Transfer 1 ml of RBC-free serum to a small glass tube, add 2 drops of concentrated formalin solution (37% w/v), mix and allow to stand for up to 2 hours.

A positive result is shown by gelling or/and whitening of the serum. Usually after 5 minutes, sometimes after 20 minutes. In the beginning of the infection, this may take up to 2 hours. A negative result is recorded when there is no gelling or whitening of the serum after 2 hours.

Reagents and staining solutions

Acetic acid solution 5%

☠ **Caution:** Acetic acid is flammable, toxic and extremely corrosive.
Acetic acid is also irritating for the eyes.
This product is always to be manipulated in a fume-cupboard, away from open flames and with extreme caution.

- distilled water: 70 ml
- concentrated acetic acid: 5 ml

Never add water to a recipient containing a concentrated acid!!! Adding even the smallest amount of water to a concentrated acid will produce an extremely violent reaction, causing the recipient to EXPLODE.
Measure 70ml of distilled water and put in a brown, glass bottle of 250 ml. Measure 30ml of concentrated acetic acid in a dry cilinder and add slowly and carefully to the water. During this process the solution will heat up (exothermal reaction)!
This solution can be kept at least 1 year at room temperature in a brown, tightly closed, glass bottle.

Acid alcohol 3%

☠ **Caution:** Ethanol is extremely flammable!
Concentrated hydrochloric acid is extremely corrosive. Hydrochloric acid fumes are toxic.
This product is always to be manipulated in a fume-cupboard and with extreme caution.

- ethanol 96%: 970 ml
- concentrated HCl: 30 ml

Put 970ml of ethanol 96% in a brown, glass bottle. Slowly and carefully add 30ml of hydrochloric acid. Mix well.
This solution can be kept indefinitely at room temperature in a brown, tightly closed, glass bottle.

Alcohol-ammonia solution

☠ **Caution:** Ethanol is extremely flammable!
Ammonia is a corrosive product. Ammonia fumes are irritating.
These products are to be manipulated in a fume-cupboard and with extreme caution.

- ethanol 70%: 250 ml
- ammonia 25%: 3 ml

Mix the ethanol 70% and the ammonia 25%.
This solution can be kept 1 week at room temperature.

Alcohol 70% solution

☠ **Caution:** Ethanol is extremely flammable!

- ethanol 95%: 730 ml
- distilled water: 270 ml

Mix the ethanol 95% and the water.
This solution can be kept at least 1 year at room temperature in a brown, tightly closed, glass bottle.

Carbol-fuchsin

☠ **Caution:** Phenol is extremely corrosive and toxic!
Ethanol is flammable!

Preparation of fuchsin-stock solution:

- alkaline fuchsin: 25 g
- ethanol 96%: 250 ml

The alkaline fuchsin (25g is one small bottle) is transferred to a brown glass bottle of 250ml. Next 250ml of ethanol 96% is added. Tightly close the bottle and shake vigorously three times on the same day. Let the solution rest during the night. If there is still a red precipitation present, some more ethanol should be added. If desired, the alkaline fuchsin can be dissolved in methanol instead of in ethanol. The bottle with the saturated stock solution doesn't have to be rinsed or washed out each time a new batch is made. From time to time a small amount of fuchsin and alcohol can be added. As long as there is a small amount of powder on the bottom of the solution, the solution is saturated.
This stock solution can be kept a few years at room temperature in a brown, tightly closed, glass bottle.

Preparation of phenol-stock solution (5% v/v):

- penol (carbol) molten cristals: 50 ml
- distilled water: 950 ml

Normally, phenol is colourless. If the phenol has a pinkish colour, it has expired and should not be used anymore.
The phenol cristals should be heated up to 45°C in a warm-water bath. Measure 50ml phenol in a cilinder (also preheated at 45°C to avoid recristallisation in the cilinder). Alternatively, use a flask of 50g phenol (Fluka 77610). The phenol is transferred into 950ml of distilled water.
This solution can be kept for some months at room temperature in a tightly closed, glass bottle.

Preparation of the workingsolution:
Mix 100 ml of filtered fuchsin-stock solution and
900 ml of phenol-stock solution 5%
This solution can be kept for at least 2 years at room temperature in a brown, tightly closed, glass bottle. Filter again before use.

Digestion-solution for biopsies

☠ **Caution:** Concentrated hydrochloric acid is extremely corrosive. Hydrochloric acid fumes are toxic. Manipulate this product with extreme caution in a fume cupboard.

- distilled water: 1000 ml
- pepsine: 20 g
- NaCl: 8,5 g
- concentrated HCl: 5 ml

Pour 1000 ml of distilled water into a brown glass bottle. Carefully and slowly add 5 ml of concentrated hydrochloric acid. Mix well. Add the pepsine and the NaCl. Mix until all is in solution.
The final solution can be kept at least 1 year at room temperature in a brown, well closed, glass bottle.

Formolated saline 10%

☠ **Caution:** Formol is corrosive and toxic. Formol-fumes are irritating for eyes and mucosa. Formol is to be manipulated in a fume-cupboard (or with open windows).

- commercialy available formol (formaldehyde 37%): 100 ml
- physiological saline: 900 ml

Mix the formol and the physiological saline well.
This solution can be kept for at least 2 years at room temperature in a brown, tightly closed, glass bottle.

Formolated water 2%

☠ **Caution:** Formol is corrosive and toxic. Formol-fumes are irritating for eyes and mucosa. Formol is to be manipulated in a fume-cupboard (or with open windows).

- commercialy available formol (formaldehyde 37%): 2 ml
- distilled water: 98 ml

Mix formol and distilled water well.
This solution can be kept at least 2 years at room temperature in a brown, tightly closed, glass bottle.

Glycerinewater

- glycerine: 300 ml
- distilled water: 700 ml

For faster clarification, use a more concentrated solution:

- glycerine: 500 ml
- distilled water: 500 ml

If desired, 10ml of malachitegreen or methyleneblue (3% solution in water) can be added per liter glycerinewater in order to boost contrast in the microscopic preparation.
This solution can be kept for at least 1 year at room temperature in a brown, tightly closed, glass bottle.

Iron-hematoxylin solution

☠ **Caution:** Ethanol is extremely flammable!

Hematoxylin stock solution :

- hematoxylin (VWR 1.15938): 10 g
- ethanol 100%: 1000 ml

Dissolve the hematoxylin powder in the ethanol in a clear glass bottle! Let the solution ripen for at least 2 weeks in sunlight before use.
This solution can be kept one year at room temperature in a clear, tightly closed, glass bottle.

☠ **Caution:** Concentrated hydrochloric acid is extremely corrosive. Hydrochloric acid fumes are toxic. Manipulate this product with extreme caution in a fume cupboard.

Mordant stock solution :

- Ammonium-iron(II) sulphate ($(NH_4)_2Fe(SO_4)_2.6H_2O$): 10 g
- Ammonium-iron(III) sulphate ($(NH_4)Fe(SO_4)_2.12H_2O$): 10 g
- concentrated hydrochloric acid (HCl 37%): 10 ml
- distilled water: 1000 ml

Dissolve the ammonium-iron(II) sulphate and the ammonium-iron(III) sulphate in about 800 ml of distilled water. Add carefully and slowly 10 ml of concentrated hydrochloric acid. Add distilled water up to 1000 ml.
This solution can be kept one year at room temperature in a, tightly closed, glass bottle.

Working-solution :

- hematoxylin stock solution: 80 ml
- mordant stock solution: 80 ml

Mix the two solutions well. The mixture will become slightly warm. Let the mixture cool down for at least 2 hours before use.
This solution can be kept one week at room temperature or for staining 200 preparations.

KOH-solution 1%

☠ **Caution:** Potassiumhydroxyde is extremely corrosive!

- potassiumhydroxyde pellets (KOH): 1 g
- distilled water: 100 ml

Dissolve the potassiumhydroxyde in the distilled water.
During this process the solution will heat up (exothermal reaction)!
This solution can be kept for 2 years at room temperature in a brown, tightly closed, glass bottle.

Lugol's staining solution according to d'Antoni

☠ **Caution:** Lugol's solution is a product containing iodine, which is very volatile and can interact with metals and other products in its close environement!
Sublimated iodine is toxic when inhaled (vapors) and in contact with the skin.

- potassium-iodine (KI): 5 g
- distilled water: 500 ml
- sublimated iodine (I_2): 7,5 g

First, the potassium-iodine is added to the distilled water. Next, the sublimated iodine is ground and the potassium-iodine solution is added little by little.
The solution can be kept unfiltered for one year at 4°C in a brown, tightly closed, glass bottle. For use, a small amount of this solution should be filtered and can be kept from this moment on for 1 month at room temperature.

Mayer's albumin

- fresh egg-white: 20 ml
- glycerin 87%: 20 ml

Mix and shake the egg-white and the glycerin vigorously by hand during at least 1 minute. Let the mixture stand for 30 minutes. A layer of foam will apear on top of the solution. Eliminate the layer of foam.
This solution can be kept 3 months at 4°C.

Methyleneblue solution

☠ **Caution:** Ethanol is extremely flammable!

- methyleneblue (powder): 25 g
- ethanol 96% : 250 ml

The methyleneblue (25g is one flask) is transferred to a brown, glass bottle of 250ml. 250 ml of ethanol 96% is added. Tightly close the bottle and shake vigorously three times on the same day. Let the solution rest during the night. If there is still a blue precipitation present, some more ethanol should be added.
If desired, the methyleneblue can be dissolved in methanol instead of in ethanol. The bottle with the saturated solution doesn't have to be rinsed or washed out each time a new batch is made. From time to time a small amount of methyleneblue and alcohol can be added. As long as there is a small amount of powder on the bottom of the solution, the solution is saturated.
This stock solution can be kept a few years at room temperature in a brown, tightly closed, glass bottle.

NaOH-solution 1%

☠ **Caution:** Sodiumhydroxyde is extremely corrosive!

- sodiumhydroxyde pellets (NaOH): 1 g
- distilled water: 100 ml

Dissolve the sodiumhydroxyde in the distilled water.
During this process the solution will heat up (exothermal reaction)!
This solution can be kept for 2 years at room temperature in a brown, tightly closed, glass bottle.

Pandy's reagent

☠ Caution: Phenol is a highly corrosive and harmful chemical. This product must be manipulated with extreme care. Phenol is also hydroscopic.

- phenol (C_6H_6O): 50 g
- distilled water: 500 ml

Crystallised phenol is normally colourless. When the colour is pink violet, it has expired and should no longer be used. Packages of 50 g are available [e.g. : Fluka 77610]
Pandy's reagent is a saturated phenol solution. Bring the phenol in a brown flask of 1000 ml. Add 500 ml of distilled water. Agitate intensely. Let rest for 1 day. Verify that enough non dissolved phenol remains. If so, filter and store in a brown flask. If all the phenol is dissolved, add 10 g phenol and wait one more day before filtering.
The solution can be kept some years in a brown, hermetically closed bottle**.**

Physiological saline

- NaCl: 8,5 g
- distilled water: 1000 ml

Dissolve the NaCl in the distilled water.
This solution can be kept a few weeks at room temperature. Check regularly for growth of bacteria or fungi: the solution should be replaced when a cloudy aspect is observed after shaking.

Picric acid solution

☠ Caution: Picric acid is flammable and explosive when dry.
Avoid shocks, frottations and dispersion of small particles.
This product is to be manipulated with extreme caution and far away from ignition sources.

Stock solution :

- picric acid ($C_6H_3N_3O_7$): 13 g
- distilled water: 1000 ml

Dissolve the picric acid in the distilled water, using a water bath at 60°C.
This solution can be kept one year at room temperature in a brown, tightly closed, glass bottle.

Working-solution :

- picric acid stock solution: 80 ml
- distilled water: 80 ml

Mix the two solutions well. This solution can be kept one week at room temperature or for staining 200 preparations.

SAF-fixative

☠ Opgepast: Acetic acid is flammable, toxic and extremely corrosive.
Acetic acid is also irritating for the eyes.
This product is always to be manipulated in a fume-cupboard, away from open flames and with extreme caution.
Formol is corrosive and toxic. Formol-fumes are irritating for eyes and mucosa.
Formol is to be manipulated in a fume-cupboard (or with open windows).

- sodium-acetate ($C_2H_3NaO_2$): 1,5 g
- distilled water: 92,0 ml
- acetic acid ($C_2H_4O_2$): 2,0 ml
- commercialy available formol (formaldehyde 37%): 4,0 ml

Mix these products in exactly the same order as described above in a dark brown flask and shake well.
NEVER add water to a recipient containing concentrated acetic acid!!! Addition of even the smallest amount of water to an acid, will produce such a strong reaction, causing the recipient to explode.
This solution can be kept at least 1 year at room temperature in a brown, tightly closed, glass bottle.

Saturated NaCl-solution

- sodiumchloride (NaCl): 250 g
- distilled water: 1000 ml

Add the sodiumchloride to the water and heat up the whole to dissolve as much of the salt as possible. The solution can be used once it has reached room temperature again.
This solution can be kept a few months at room temperature.

Saturated $ZnS0_4$-solution

- zinc-sulphate ($ZnS0_4$): 330 g
- distilled water: 1000 ml

Add the zinc-sulphate to the water and heat up the whole to dissolve as much of the salt as possible. The solution can be used once it has reached room temperature again.
This solution can be kept a few months at room temperature.

Sörensen buffer solution

Stock solution :

KH_2PO_4 : 9,078 g/l in distilled water (solution A)
$Na_2HPO_4.2H_2O$: 11,876 g/l in distilled water (solution B)

Both stock solutions can be kept several weeks at 4°C in dark, glass bottles. Check regularly for growth of bacteria or fungi: the solution should be replaced when a cloudy aspect is observed after shaking.

Working-solution :

Use	pH	Volume solution A (in ml)	Volume solution B (in ml)	Volume distilled water (in ml)
Haematology	**6,6**	64,0	36,0	900
Haematology	**6,8**	50,0	50,0	900
Malaria	**7,0**	39,0	61,0	900
Malaria	**7,2**	28,0	72,0	900
	7,4	19,2	80,8	900
Trypanosomes	**7,6**	13,0	87,0	900
	7,8	8,5	91,5	900
Malaria / Microfilaria	**8,0**	5,5	94,5	900

The Sörensen buffering-solution can be replaced by buffering-pills of pH 7,2 (available with VWR (formerly known as *Merck*) nr.9468).
According to instructions: 1 pill per liter water (distilled or filtered).
According to our experience: depending on the quality of the used water, one pill can buffer up to 10 liters of water (lowering the price in a spectacular way!). This should however always be checked by staining a few smears and evaluating the results.
This buffering-solution can be kept for one month at room temperature.

Sulfatation reagent

☠ **Caution:** Diethylic ether is extremely flammable and will ignite and explode quickly when in contact with a flame or spark. Manipulate this product far away from ignition sources. Recipients, once opened, are to be kept in a high (dispersion of fumes) and cool place in the lab. As diethylic ether is very volatile, recipients should be closed well and hermetically. A recipient with diethylic ether should NEVER be kept in the refrigerator: diethylic ether-fumes will build up and the whole may explode when opening the fridge (spark of refrigerator-light).
Recipients in use should never be placed together in a cupboard. Avoid having large amounts of diethylic ether in the lab.
Sulphuric acid is extremely coorosive. Sulphuric acid fumes are toxic. This product is to be manipulated with extreme caution in a fume cupboard (or with open windows).

- diethylic ether: ±140 ml
- concentrated sulphuric acid : 100 ml
- distilled water: 40 ml

Mix thoroughly 140 ml of diethylic ether and 40 ml of ditilled water in a decantation ampoule. Wait until two layers are formed. Eliminate the lower layer (water) as well as some ml of the upper layer (ether-hydrate) to clean the tubing. Transfer the ether hydrate into an Erlenmayer-flask, which has been placed **in ice**. Add carefully (drop by drop) the sulphuric acid, while shaking the Erlenmayer-flask.
This solution can be kept one month in a brown, tightly closed, glass bottle. The solution should be discarted when two layers are observed.

Toluidine blue O solution

☠ **Caution:** Ethanol is extremely flammable!
Toluidine blue O is toxic when inhaled, in contact with skin or when ingested.
Toluidine blue O fumes are irritating for the eyes and mucosa.
Manipulate this product in a fume cupboard (or with open windows), using gloves.
Concentrated hydrochloric acid is extremely corrosive. Hydrochloric acid fumes are toxic. Manipulate this product with extreme caution in a fume cupboard.

- toluidine blue O: 320 g
- distilled water: 60 ml
- concentrated hydrochloric acid (HCl 37%): 2 ml
- absolute ethanol: 140 ml

Dissolve the toluidine blue O powder (e.g. Sigma T0394) in the distilled water, using a magnetic stirrer. Very carefully add the 2 ml of hydrochloric acid (while stirring). Then **slowly** add the ethanol.
This solution can be kept several months in a brown, tightly closed, glass bottle.

Trichloroacetic acid 30% (w/v)

☠ **Caution:** Trichloroacetic acid is a strongly corrosive and deliquescent chemical with an irritating vapour. Handle with care in well ventilated area (or in a fume-cupboard)

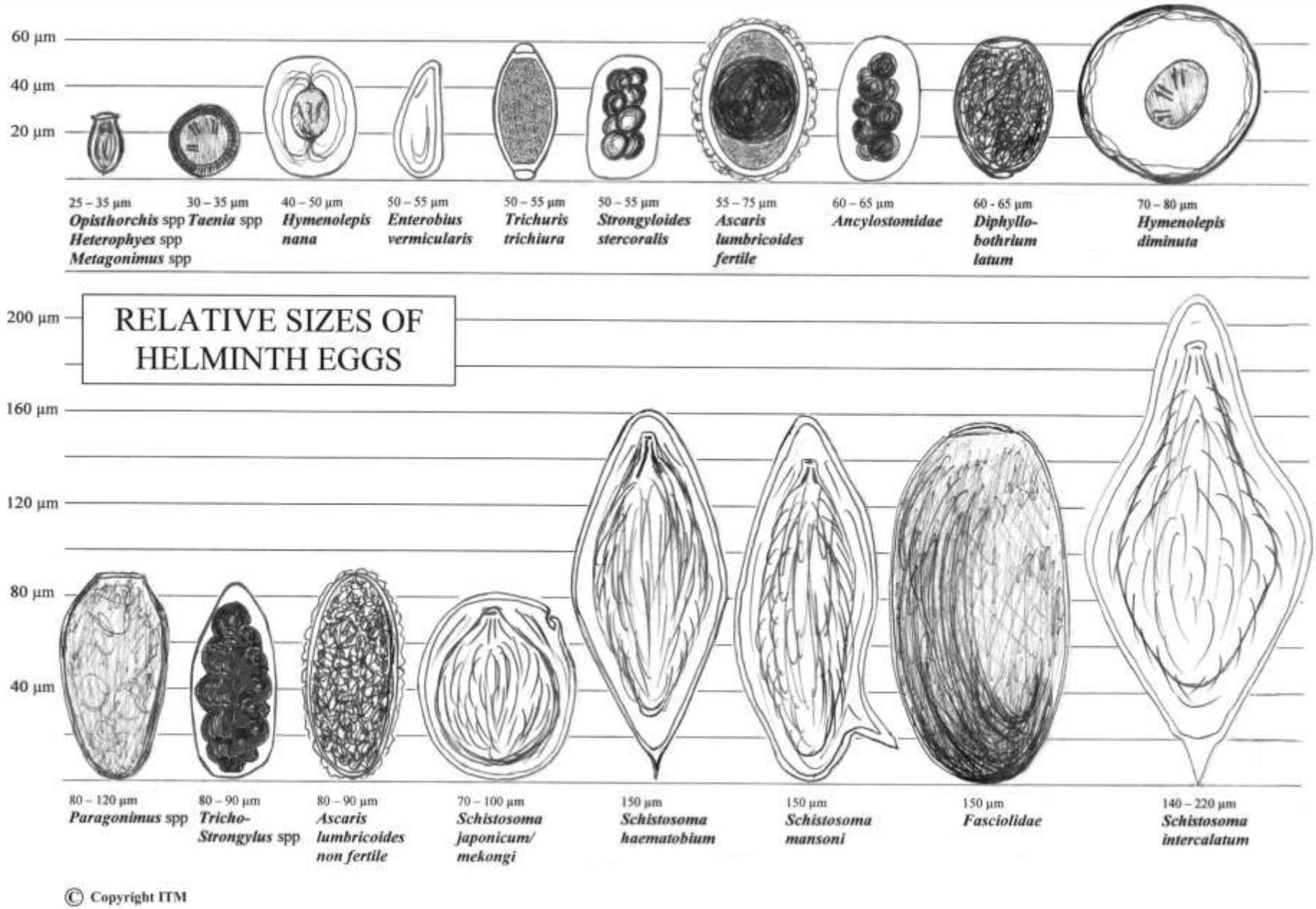
- trichloroacetic acid ($C_2HCl_3O_2$): 30 g
- distilled water: 100 ml

Dissolve the trichloroacetic acid in the distilled water.
The solution can be kept some weeks in the refrigerator (4°C).

Abbreviations

µl	microliter(s)
µm	micrometer(s)
Ag	Antigen(s)
Ab	Antibody (~ies)
e.g.	*ex grata*, for instance
i.e.	*it est*, this is
CAA	Circulating Anodic Antigen
CATT	Card Agglutination Test for Trypanosomiasis (*Trypanosoma b. gambiense*)
CCA	Circulating Cathodic Antigen
CDC	Centers for Disease Control and Prevention
CSF	CerebroSpinal Fluid
DEC	DiEthylcarbamazine Citrate
EDTA	Ethylene-Diamine Tetra-Acetic acid (anticoagulentium)
EIA	Enzyme ImmunoAssay
ELISA	Enzyme Linked ImmunoSorbent Assay
EN	English
ES	Spanish
ES-Ag	Excretor/Secretor-Antigen
ESR	Erythrocyte Sedimentation Rate
FR	French
g	centrifugal force
g	gram(s)
GGT	Gamma-Glutamyl-Transpeptidase
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
Ht	Haematocrit
IFAT	Immuno-Fluorescent Antibody Test
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect HemAgglutination
KI	Potassium-iodine
KIVI	Kit for In Vitro Isolation (*Trypanosoma cruzi*)
KOH	PotassiumHydroxide
LDH	LactateDehydroGenase
LFA	Lateral Flow Assay
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
NaCl	SodiumChloride
NaOH	SodiumHydroxide
NL	Dutch
PCR	Polymerase Chain Reaction
QBC	Quantitative Buffy Coat
RBC	Red Blood Cell(s)
rpm	revolutions per minute
SAF	Sodium acetate – Acetic acid – Formalin (fixative)
sp.	species (only the mentioned one)
spp.	species (all the ones, belonging to a certain Genus)
TBC	Tuberculosis
h	Hour(s)
WBC	White Blood Cell(s)
WHO	World Health Organization
ZnSO4	Zinc-sulphate

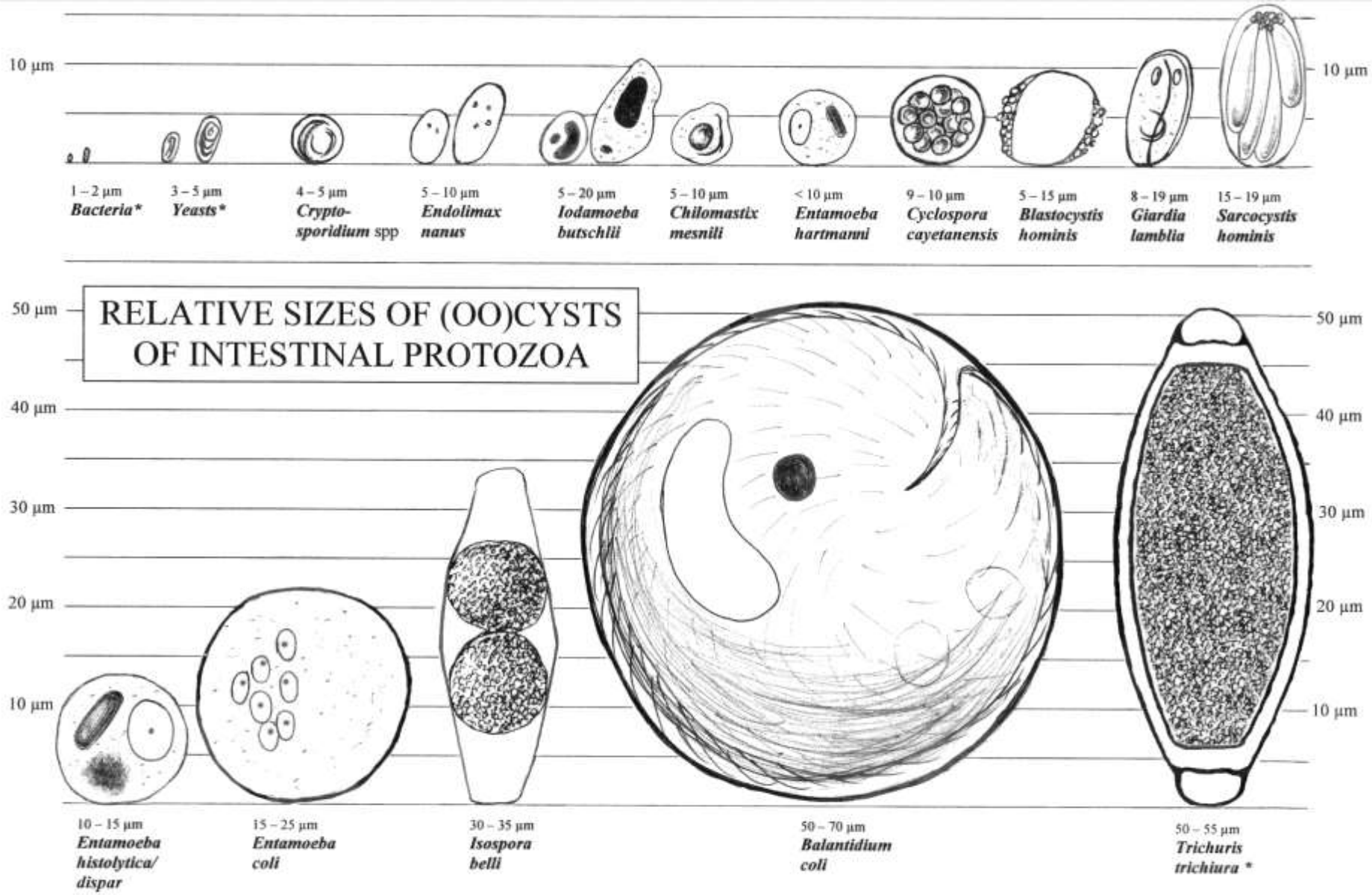
Schematic overview of the helminth eggs



Some useful parameters concerning parasitic helminths (filaria not included)

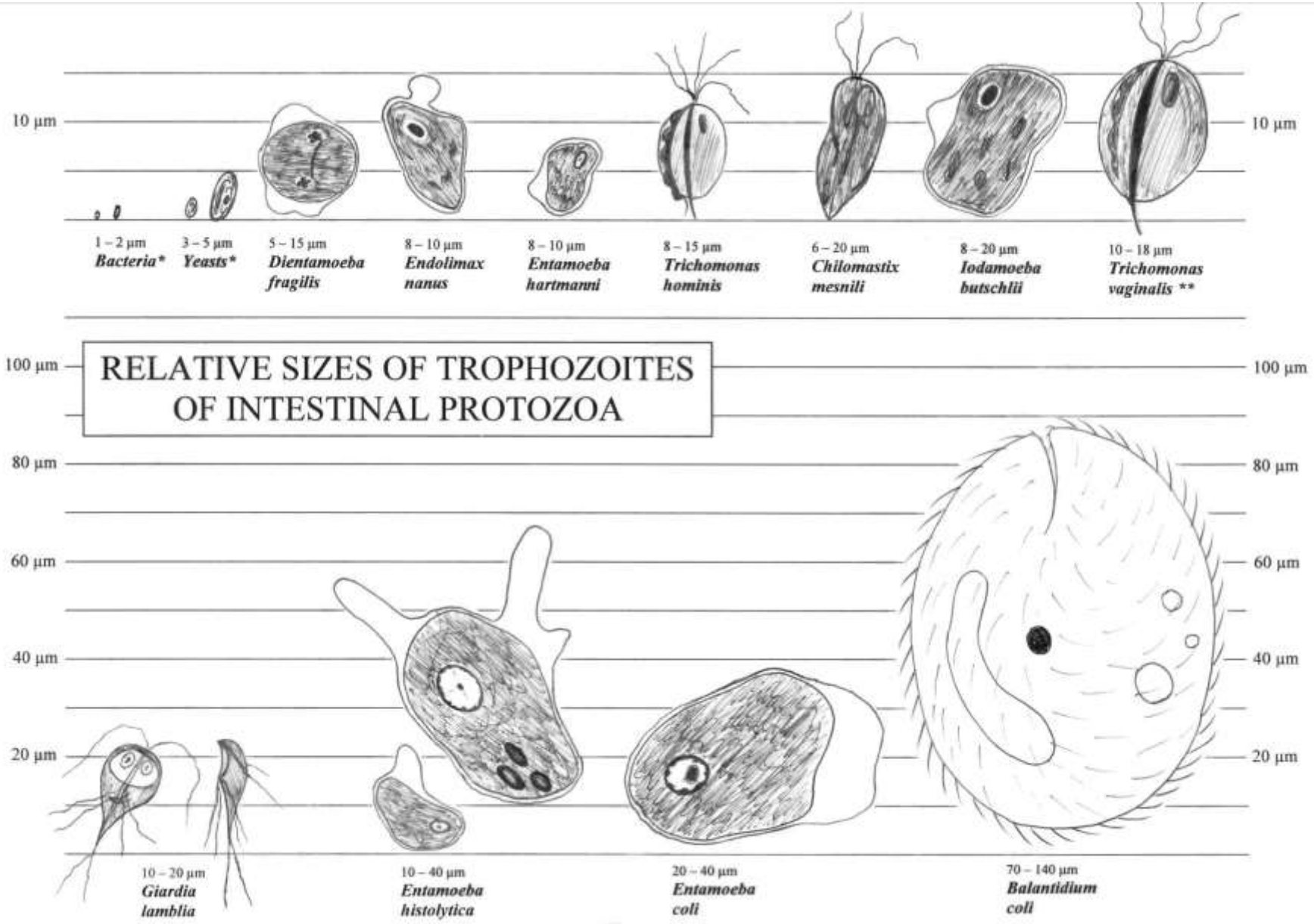
Organism	Time between initial infestation and appearance of fist eggs/larvae (maturation)	Estimation of daily egg-production (per adult/female)	Estimated lifespan
Ancylostoma duodenale	15 - 40 days	5.000 - 22.000	1 - 9 years
***Anisakis* spp.**			
Ascaris lumbricoides	2 - 3 months	200.000	1 - 3 years
Capillaria aerophila			+/- 1 year
Capillaria hepatica	2 - 3 weeks		1 - 4 months
Capillaria philippinensis			
Clonorchis sinensis	1 - 4 weeks	1.000 - 4.000	up to more than 25 years
Diphyllobothrium latum	30 - 45 days	35.000 – 1.000.000	up to 30 years
Echinococcus granulosus	Not applicable	Not applicable	Not applicable
Enterobius vermicularis	+/- 3 weeks	+/- 500	up tot 55 days (but auto-infestation)
Fasciola gigantica	12 - 15 weeks	worms usually sterile	up to more than 11 years
Fasciola hepatica	11 - 17 weeks	25.000 (only few arrive in faeces)	up to more than 25 years
Fasciolopsis buski	12 - 20 weeks	+/- 16.000	+/- 6 months
Hymenolepis diminuta	+/- 20 days		5 - 7 weeks
Necator americanus	15 - 40 days	3.000 - 6.000	4 - 20 years
***Paragonimus* spp.**	2 - 3 months		10 - 20 years
Schistosoma haematobium	54 - 84 days	20 - 300	3 - 7 years
Schistosoma intercalatum	50 - 60 days	150 - 400	
Schistosoma japonicum	30 days	1.500 - 3.500	up to more than 25 years
Schistosoma mansoni	25 - 60 days	100 - 300	2 - 18 years
Schistosoma mekongi	30 - 60 days	1.500 - 3.500	up to more than 25 years
Strongyloides stercoralis	2 - 3 weeks		short (but auto-infestation)
Taenia saginata	10 - 12 weeks	up to 2.000.000	up to more than 35 years
Taenia solium	5 - 12 weeks		up to more than 25 years
Toxocara canis		Not applicable	
***Trichinella* spp.**		Not applicable	up to 4 months
***Trichostrongylus* spp.**	3 - 5 weeks		
Trichuris trichiura	30 - 90 days	3.000 - 20.000	1 - 4 years (20 years ?)
Hymenolepis nana	+/- 20 days		some months

Schematic overview of (oo)cysts of intestinal protozoa



* Shown only for comparison.

Schematic overview of trophozoites of intestinal protozoa



* Shown only for comparison.

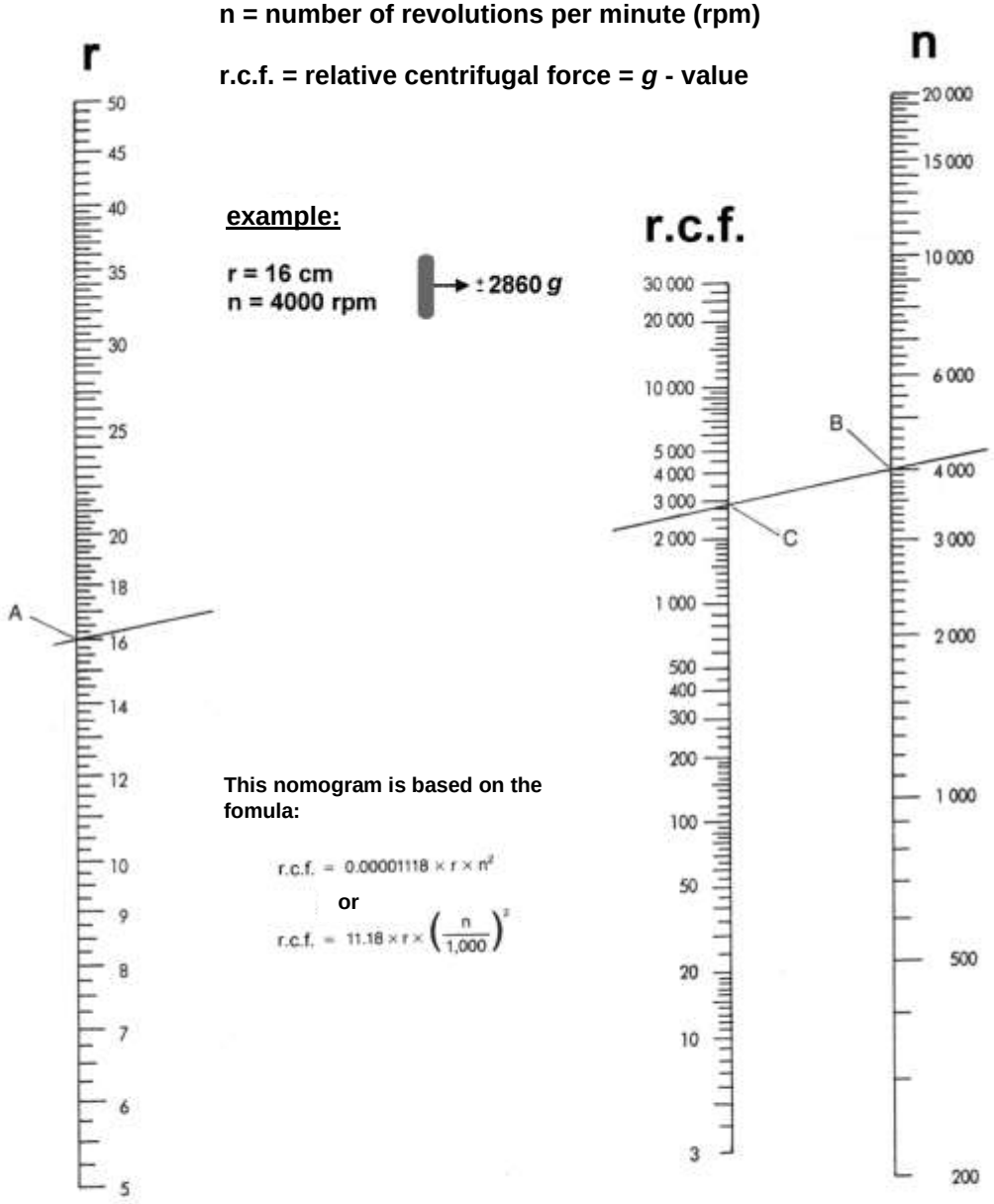
** Found in vaginal discharge or urine.

Nomogram

r = radius of the rotor (in cm)

n = number of revolutions per minute (rpm)

r.c.f. = relative centrifugal force = *g* - value



example:

r = 16 cm
n = 4000 rpm
→ **± 2860 *g***

This nomogram is based on the fomula:

$$r.c.f. = 0.00001118 \times r \times n^2$$

or

$$r.c.f. = 11.18 \times r \times \left(\frac{n}{1{,}000}\right)^2$$

MORPHOLOGY OF BLOOD CELLS IN A MAY-GRÜNWALD-GIEMSA STAINED BLOOD FILM

CELL TYPE	SIZE	NUCLEUS			CYTOPLASM		
GRANULOCYTES	µm	FORM	COLOR	CHROMATIN STRUCTURE	QUANTITY	COLOR	GRANULES
LEUKOCYTES : POLYMORPHONUCLEAR GRANULOCYTES							
IMMATURE NON SEGMENTED NEUTROPHILS "Band forms" or "S" shaped	12 – 15	Horseshoe, Central curvature is maximum a third part of the width of the lobes [1]	Clear blue purple	Strands of fine chromatin	abundant +++	Dusty rose (=very small granules)	small granules, light purple or violet Not always present
SEGMENTED NEUTROPHILS	12 – 15	2 to 5 lobes [2]	Deep blue purple	Rather thick and coarse	+++	Rose	Small granules, Pink or pink mauve
EOSINOPHILS	12– 15	Usually a bi-lobed nucleus	Blue purple	Rather thick and coarse	+++	Rose	Many large, uniform granules, red orange
BASOPHILS	11 – 13	Hardly visible lobes, not well separated (polymorph)	Blue purple	Rather thick and coarse, covered by granules	+++	Light rose	Very large, well separated, variable granules Deep purple Small in number

[1] Left deviation of the Arneth formula: an increase over 16 % of non segmented neutrophils, yet immature forms, occurring in inflammations, but also in stress conditions,… 2 to 5 segmented neutrophils are the major fraction of the neutrophils in a normal leukocyte type.

[2] Right deviation of the Arneth formula: in contrast with the left deviation, where segmented cells are rarely seen, this image shows hyper-segmented neutrophils, with 5 or more lobes. A hyper-segmentation is characteristic for megaloblastic anaemia. In the early phase, more than one neutrophil with 6 lobes per 100 granulocytes is found.

CELL TYPE	SIZE	NUCLEUS			CYTOPLASM		
AGRANULOCYTES	µm	FORM	COLOR	CHROMATIN STRUCTURE	QUANTITY	COLOR	GRANULES
LEUKOCYTES : MONOMORPHONUCLEAR AGRANULOCYTES							
SMALL LYMPHOCYTES	7 -10	Round or oval Or slightly indented	Deep purple	Big clumps of intensely stained chromatin	(-) or +	Sky blue (Often absent)	
LARGE LYMPHOCYTES	10 – 15	Round or oval Or slightly indented	Red, purple	Clumps of deep stained chromatin and other clumps which are less intensely stained	++	Sky blue	Absent or a few granules azurophils (rose violet)
MONOCYTES	15 – 25	Round, oval, indented or bean form	Blue to slightly violet	Fine, spongy like	+++ Vacuoles often demonstrable.	Grey or blue grey	Very fine granules (dusty like), azurophils (rose violet)
ERYTHROCYTES	6,7 – 7,7				Biconcave discus shape	rose	none
TROMBOCYTES	1,5 – 2 (5)				Slightly blue		Reddish

Some useful internet-sites

- Site for human parasitology (CDC):

  http://www.dpd.cdc.gov/dpdx

- Site for human parasitologie (Carlo Denegri Foundation - Italy):

  http://www.cdfound.to.it/html/atlas.htm

- Site for veterinary parasitology (Merck):

  http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/22600.htm

- Site for human parasitology (medical chemical corporation):

  http://www.med-chem.com/Para/Index.htm

- Site for mycology:

  http://www.doctorfungus.org/index.htm

- Site for common laboratory-techniques – links to parasitology, haematology etc. (Australia):

  http://www.hoslink.com/malaria.htm

- Site for malaria-information (Australian Government):

  http://www.rph.wa.gov.au/malaria.html

- Site for new lab-tests:

  http://www.finddiagnostics.org

- Site for human parasitology (in dutch):

  http://www.parasitologie.nl

- Site for veterinary parasitology:

  http://www.wormers-direct.co.uk

Instituut voor Tropische Geneeskunde
Institut de Médecine Tropicale
Institute of Tropical Medicine
Instituto de Medicina Tropical

Nationalestraat, 155
B – 2000 Antwerpen

Stichting van Openbaar Nut 0410.057.701

POSTGRADUATE IN TROPICAL MEDICINE AND INTERNATIONAL HEALTH
MODULE 1&2
CLINICAL & BIOMEDICAL SCIENCES OF TROPICAL DISEASES

Practical notes

TROPICAL BACTERIOLOGY

PHILIPPE GILLET, IDZI POTTERS, HILDE DE BOECK, JAN JACOBS
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INTRODUCTION

The direct examination of a clinical sample that contains bacteria is usually not sufficient for the species identification of the disease causing bacteria; therefore, culture associated with a series of biochemical tests is necessary.
Specific problems of bacterial microscopy are causing these restrictions:

- They have very small sizes : width 0,2 – 2 µm length 1 - 10 µm ; this means that the microscopical examination is at the limit of its (optic resolution) possibilities.
- The number of different species is tremendous, while their morphological differences are little and subtle: cocci, rods, vibrios, spirila, spirochaetes
- Bacteria are colourless (necessitate specific staining as Gram, Ziehl-Neelsen, Giemsa,…) [**Gram affinity** is a characteristic that depends on a chemical component of the cell wall: peptidoglycane. If this layer contents a large amount of it, the bacteria are Gram positive, Gram negative bacteria contain a small amount of peptidoglycane].
- There is no morphological difference between pathogen and non-pathogen bacteria (symbionts, saprophytes, commensals, pathogens, opportunists). Samples are often contaminated with environmental microorganisms. Special attention should always be paid for the sample taking.
- Several species of bacteria are able to change their shape, especially after growing on artificial media, in immune depressive persons, as different strains of bacteria (Organisms which show variation in shape are described as pleomorphic).
- The virulence of bacteria depends on :
    - The strain and serotype of the bacteria
    - Its transmission route
    - Their final localisation.
    - The amount of invading bacteria.
    - The state of health of the infected person.
- Infections in non sterile localisations (intestine, nasofarynx, vagina, …), give diagnostic problems.

In resource-restricted settings, such as in district hospitals, where cultures are impossible, samples of patients with suspected important symptoms can be shipped to reference laboratories.
In spite of these limited feasibilities, microscopical examination of stained smears with appropriate staining methods **(Gram, Ziehl-Neelsen, Giemsa, ...)** can sometimes supply a rapid and complete diagnosis for samples from normally sterile locations or for some particular samples, lepra, anthrax, some forms of borreliosis,…). Sometimes a partial diagnosis can be delivered: the distinction between gram positive cocci and gram negative rods. (example: Urinary tract infections) or a differential diagnosis: bacterial versus viral, versus malaria, versus sleeping disease infections...(example in meningitis). In some specific situations, an almost precise diagnosis can be given :

- Sputum for infectious pulmonary tuberculosis identification
- Cerebro Spinal fluid (CSF) for bacterial or fungal identification of meningitis
- Urethral discharge for acute gonococci infection in male subjects
- Blood for some forms of borreliosis (recurrent fever)
- Lepra, anthrax…

It can enable the morphology, relative sizing, capsule and arrangement of micro-organisms to be seen clearly, and also assist in the detection of cells, especially pus cells.

*Many bacteria secrete around themselves a polysaccharide substance often referred to as a slime layer. This may become sufficiently thick to form a definite capsule around the organism. This **capsule** increases the virulence of an organism by resistance against phagocytosis by host cells.*
Special techniques or stainings s.a. India ink or Giemsa staining can show these capsules. The serotyping of several bacteria is based on their capsule (Haemophilus influenza b…).

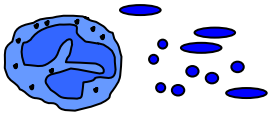
As conclusion, we can state that microscopy for bacteriological purposes can often deliver rapid and important information from which indispensable treatment can be started in order to control life threatening infections.
Some easy serological tests can also provide important information (syphilis, bacterial meningitis …).

COMMON TECHNIQUES IN BACTERIOLOGY

1. METHYLENE BLUE STAIN:

PRINCIPLE :

The methylene blue stain is a simple technique, staining everything blue. Bacteria and cells are visualised without enabling differentiation of bacteria according to their Gram affinity **(Gram positive / Gram negative).** It can be applied for searching bacteria in spinal fluid or in urethral pus (to find intra and/or extra cellular diplococci). It can be an alternative for the Gram stain in cases when the dyes and products for the Gram are not available. Another advantage of this staining is that it is almost always successful.



REAGENTS AND MATERIAL :

Methanol, immersion oil, methylene blue (used for Ziehl stain).
Microscope slides, diamond marking pen, alcohol 70 % (or cresol) sand flask, inoculation wire loop, Bunsen burner or spirit lamp, filter paper, funnel, staining rack to hold smear slides, rinsing water, slide rack to dry smears in the air, microscope.

METHOD :

1. Register the sample in the lab register and note the patient's number with the marking pen on a new slide.
2. Subsequently the slide is gently flamed by moving it three times through a hot flame.
3. Mechanical decontamination in sand and disinfectant by drenching the wire loop in the alcohol sand flask.
4. Then flame the inoculation loop until red-hot and let it cool.
5. Carefully take out a little bit of sample with the wire loop and smear it on the slide in an as thin as possible layer.
6. Decontaminate again mechanically by drenching the wire loop in the alcohol sand flask and flame it until red-hot in order to kill all bacteria.
7. Let the smears dry in the air for at least 15 minutes protected against insects.
8. The dry smear is fixed by covering it with pure methanol [or by passing the slide 3 times through a hot flame on the opposite side of the sample smear.
9. Allow the slide to dry or to cool.
10. Cover the slide completely with methylene blue during 1 minute and 30 seconds.
11. Rinse the slide with (clean tap or filtered) water.
12. Let dry in the air.
13. Observe the slide microscopically (with immersion oil, objective 100 x, eyepiece 10 x)

TYPES OF ANSWERS

Presence of :

Quantity:	Rare, few, numerous bacteria.
Form of the bacteria:	Thick or small cocci, short or long bacilli, with rounded ends.
Disposition of the bacteria:	Isolated, in pairs (= diplo-), in chains, or in groups.
Localisation of the bacteria:	Intra- or extracellular.
Particularities:	Capsules.
Cells:	**P**resence and number of pus (inflammatory, polynuclear cells), number/field, epithelial cells

negative examination

2. INDIAN INK STAIN

PRINCIPLE:

This technique enables the visualisation of the encapsulated forms of *Cryptococcus neoformans*. The sample (e.g. a drop of the centrifuged sediment of a spinal fluid) is mixed with an equal drop of Indian ink and microscopically examined. The capsule is seen as a lightening aureole around the fungus. Generally, the round or oval yeast form is found, sometimes developing buds of short mycelium, containing greyish granulation.

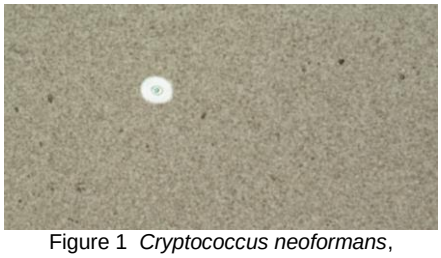


Figure 1 *Cryptococcus neoformans*,
Spinal fluid, Indian ink stain, 400 x

REAGENTS AND MATERIAL :

Indian ink, microscope slides, cover slides, diamond marking pen, inoculation wire loop or Pasteur pipette, Bunsen burner or spirit lamp, microscope.

METHOD :

1. Register the sample in the lab register and note the patient's number with the marking pen on a new slide.
2. Bring a drop of Indian ink on a slide.
3. Mechanical decontamination in sand/disinfectant by drenching the wire loop in the alcohol sand flask.
4. Then flame the inoculation loop until red-hot and let it cool.
5. Transfer an appropriate portion of the specimen (centrifuged sediment of the spinal fluid) near to the ink drop and mix them.
6. Decontaminate again by drenching the wire loop in the alcohol sand flask and flame it until red-hot in order to kill all bacteria.
7. Cover the mixture with a cover slip.
8. Examine under the microscope (objective 40 x, eyepiece 10 x)

TYPES OF ANSWERS

Presence of :

Quantity : Rare, few, numerous fungi.
Particularities : Round or oval capsules, pseudomycelial large yeast cells with thick capsules

Negative examination

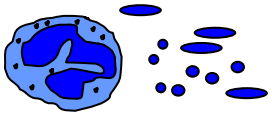
3. GRAM STAIN

PRINCIPLE :

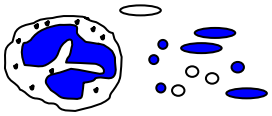
The gram is a differential stain that enables classification of the bacteria into two large groups, the Gram positive and the negative group (based on their reaction to the stain and according to differences in the structure of their cell walls).
The Gram staining procedure is based on the fact that Crystal Violet stains the bacterial peptidoglycan cell envelope. The Gram positive bacteria have a thick peptidoglican cell envelope and no membrane at the outside of their cell envelope. Gram negative bacteria have a lipopolysaccharide on the membrane that covers the cell envelope and this membrane prevents to stain by Crystal Violet dyes. Gram negative will be decolorised and are counterstained.
Even in modern laboratories Gram staining still has an important role and is always used before starting a series of diagnostic cultures.

For the Gram stain 4 reagents are used :

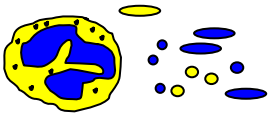
- The **Cristal Violet** dye is the primary stain, which stains everything in the smear violet/deep blue.



- Diluted **Lugol** or Gram's iodine acts as a, yellow brown, mordant solution that causes the crystal violet to penetrate and adhere to the gram-positive organisms forming an alcohol dissolvable complex.
- **Alcohol 96 % or Alcohol/Acetone** will decolorize or not certain bacteria according to their cell membrane composition.



- **Safranin** is the counter-stain that stains everything in the smear that has been decolorized: pus cells, mucus, Gram negative organisms. The Gram-negative organisms will stain a much deeper pink than the pus cells and the mucus cells will stain even lighter pink. Instead of Safranin, also neutral red or fresh diluted Fuchsin from Ziehl (1ml + 9 ml water, clean or filtered) can be used.



MATERIAL :

Microscope slides, diamond marking pen, alcohol 70 % (or cresyl) sand flask, inoculation wire loop, Bunsen burner or spirit lamp, filter paper, funnel, staining rack to hold smear slides, rinsing water, slide rack to dry smears in the air, microscope, Methanol, immersion oil.

METHOD :

1. Register the sample in the lab register and note the patient's number with an inerasable marker on a new slide.
2. Flame a new slide by passing it 3 times through a hot flame.
3. Mechanical decontamination in sand and disinfectant by drenching the wire loop in the alcohol sand flask.
4. Then flame the inoculation loop until red-hot and let it cool.
5. Before starting, make sure that all reagents, as well as the squirt-bottle of water, are easily accessible because there is not enough time to retrieve them during the staining procedure.

6. Carefully take out a little bit of sample with the wire loop and smear it on the slide in an as thin as possible layer.

7. Decontaminate again mechanically by drenching the wire loop in the alcohol sand flask and flame it until red-hot in order to kill all bacteria. Subsequently the smear is gently flamed by moving it three times through a hot flam

8. Let the smears dry in the air for at least 15 minutes protected against insects.

9. The dry smear is fixed by covering it with pure methanol or by passing the slide 3 times through a hot flame on the opposite side of the sample smear.

10. Allow the slide to dry or to cool.

11. Place the slide in horizontal position on a rack. Cover the complete smear with **Crystal Violet** solution for 1 minute and 30 seconds.

12. Rinse with tap (or filtered) water until no more stain comes loose from the slide. Tip off all the water.

13. Flood the slide with the **iodine** solution. Let it stand for 1 minute and 30 seconds.

14. Rinse with tap water until no more stain releases from the slide. Tip off all the water and immediately proceed to the next step. At this point, the specimen should still be blue-violet.

15. **Alcohol-acetone**: decolorize during 2 to 30 seconds. This is the critical phase of the staining process. A few drops of the decolorizer liquid are poured on the smear while moving the slide with the other hand between thumb and finger. Do not decolorize too long. De decolorization time depends on the thickness and the nature of the smear (urine, very short,– pus, long). The decolorization continuous until the specimen is rinsed (step 16).

16. The slide is rinsed with tap (or filtered) water to stop the decolorization.

17. The final step involves applying the counterstain, **safranin**. Flood the slide with the dye as was done in steps 11 and 13. Let it stand for 1 minute and 30 seconds.

18. Rinse with tap (or filtered) water to remove the excess of dye.

19. Allow the slide to air dry or blot it gently with bibulous paper. Do not rub the smear!

20. View under the microscope not before complete drying of the slide. Apply a drop of immersion oil and use objective 100x, eyepiece 10x.

TYPES OF ANSWERS

Presence of :

Quantity:	Rare, few, numerous bacteria. Give a semi-quantification.
Gram affinity:	Gram positive or gram negative.
Form of the bacteria:	Thick or small cocci, short or long bacilli, with rounded ends.
Disposition of the bacteria:	Isolated, in pairs (= diplo-), in chains, or in groups.
Localisation of the bacteria:	Intra- or extra cellular.
Particularities:	Capsules.
Cells:	Presence and number of pus (inflammatory, polynuclear) cells, number/field, epithelial cells

Negative examination

INTERPRETATION OF THE GRAM STAINING :

The gram staining is a differential staining which makes a distinction between bacteria with a different cell wall. So Gram positive bacteria will not be decolorized by alcohol (-acetone) and they will retain the crystal violet, while Gram negatives will be decolorized and are counterstained by safranin in red.
Also the different inflammatory cell elements show a specific Gram affinity. The nucleus is gram positive, (purple), while the cytoplasm is gram negative (pink or red). This particularity offers us the opportunity to check the quality of the gram stain and to judge the Gram affinity of bacteria in the proximity of these well-stained cells.
The essential, and at the same time critical action of the Gram staining takes place at the moment of the decolordecolorization and is executed often improperly. Nevertheless, it can be possible to interpret a not so very well realised staining:

- If there was too little decolorization, everything will remain purple, also the cytoplasm of the WBC. The purple bacteria, found in this area, might be really Gram positive, or Gram negative bacteria which are not decolorized. **The pink bacteria are real Gram negatives**.

- If we decolorize too strongly, also the nuclei of the polynuclear WBC will be decolorized and stained pink during the counter staining. The pink bacteria that are near such cells might be real Gram negative bacteria or decolorized Gram-positive bacteria. **The purple bacteria here are real Gram positives.**

False positive Gram reactions

Gram negative bacteria may remain unstained and keep the purple colour.
This can be caused by :

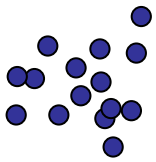
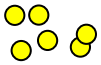
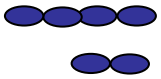
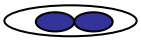
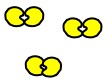
- Preparation of the smear from an old culture or sample, or too thick smear.
- Improper application of the procedure. Too short decolorization time or fixation time.
- Improper composition of the reagents or expired reagents. Too weak decolorization reagent

False Gram negative organisms

Bacteria can lose their ability to retain crystal violet and stain Gram negatively

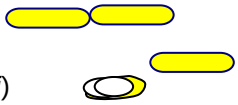
- Cell wall damage due to antibiotic therapy
- Cell wall damage due to excessive heat fixation of the smear
- Cell wall damage due to improper storage or transport of the sample
- Too old samples or too old cultures
- Use of an iodine solution which is too old (yellow instead of brown in colour), and through that not acting as an effective mordant
- Too short iodine fixation time
- Over-decolorization of the smear

CLASSIFICATION OF BACTERIA BASED ON MORPHOLOGY AND GRAM AFFINITY

GRAM POSITIVE Bacteria (Deep Purple / Bleu)	GRAM NEGATIVE Bacteria (Pink / Red)
Cocci in groups	
round shaped, in bunches of grapes  Examples : *Staphylococcus aureus* *Staphylococcus saprophyticus* …	
Cocci in chains	**Diplococci**
Oval, In pairs or in chains.  Examples : *Streptococcus pyogenes* (β hemolytic) [A][1] *Streptococcus agalactiae* (β hemolytic) [B] *Enterococcus faecalis* [D] … In pairs, with a capsule  Examples : *Streptococcus pneumoniae* (α hemolytic)	Diplococci, kidney-shaped, or coffee bean form Intra- or extra cellular.  Examples : *Neisseria meningitidis* *Neisseria gonorrhoeae* Other *Neisseria* *Moraxella (Branhamella) catarrhalis*

[1] [ ] Serogroups of *Streptococcus* spp., classification based on Lancefield polysaccharides antigens from their cell wall. *S. pneumoniae* does not possess these antigens.

CLASSIFICATION OF BACTERA BASED ON THEIR MORPHOLOGY AND THEIR GRAM AFFINITY

GRAM POSITIVE RODS (Deep purple / Blue)	GRAM NEGATIVE RODS (Pink / Red)
Thick rods	**Thick rods**
Thick bacilli with rounded ends Forming spores **Rounded edges**  Examples : **Bacillus** (*Bacillus cereus, Bacillus subtilis*,…) **Clostridium** *Clostridium tetani,*,(drum- stick bacillus) *Clostridium difficile, Clostridium botulinum, Clostridium perfringens*… … **Angular**  Examples : *Bacillus anthracis*	Thick round off rods, short or long Sometimes bipolar (*E. coli*)  Examples : **Enterobacteriaceae** (*E. coli, Shigella, Salmonella, Citrobacter, Klebsiella, Yersinia, Proteus,*…) **Pseudomonas** (*Pseudomonas aeruginosa*,…) **Borrelia** …
Medium sized rods	**Medium sized rods**
 Lactobacilli : medium size, regular shape, forming branching chains Examples: *Lactobacillus acidophilus*	Medium size, comma shape polymorphic (Gram variable)  Examples : *Vibrio cholerae*
Small rods	**Small rods**
(small, granulated, irregular) Pleomorphic, non-motile rods  **« Coryneforms »** Examples *Listeria monocytogenes* *Gardnerella vaginalis* *Bacteroïdes* *Mycobacterium* *Corynebacterium diphteriae* …	Coccobacilli, Thin, polymorphic.  **« Haemophilus like »** Examples : *Haemophilus influenzae,* *Haemophilus ducreyi* *Brucella* *Bordetella* …

4. HOT ZIEHL-NEELSEN STAIN :

PRINCIPLE :

The Ziehl-NEELSEN stain is a specific stain for mycobacteria based on their resistance to decolorization by alcohol-acid after staining with an Aryl-methane dye such as basic Fuchsin. This resistance is due to the presence of a lipids layer on their cell surface.

MATERIAL :

Microscope slides, diamond marking pen, alcohol or cresyl sand flask, wire loop, Forceps with (denaturised) alcohol drenched cotton or Bunsen burner or spirit lamp, for heating the fuchsin dye. Filter paper, funnel, staining rack to hold smear slides, microscope, immersion oil.

REAGENTS

- Carbol - **Fuchsin** : must be of the best quality.
  The best concentration is of 1 % Fuchsin in 5 – 6 % phenol
- **Acid**-**Alcohol** for the decolorization : a very strong decolorizer : sulphuric acid 20 – 25 % in water or HCl 3 % in alcohol 70 % which may be of technical quality.
- **Methylene blue** 0,1 % in water

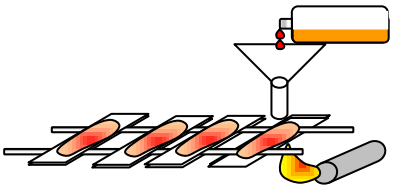
Each new lot (surely of fuchsin) should be tested on its function and its purity with a positive and a negative sputum sample.

- Pure water: distilled or filtered. Tap water, or other water, often contains saprophytic mycobacteria, which cannot be distinguished microscopically from pathogen mycobacteria.
- Denaturised alcohol
- Dettol

METHOD

1. Put the fixed slides on horizontal bars. Avoid contact between the slides in order to avoid cross contamination and to avoid that

2. Then cover the slide with filtered alkaline carbol – fuchsin. The whole smear must be covered with the dye solution.

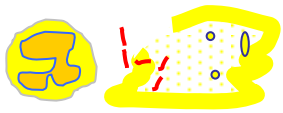


3. Heat each slide slowly until it is steaming (white vapours ascending), but avoid boiling at any time. Repeat this intermittent heating two or three times.
4. Let stain for 5 minutes. A longer time intensifies the colouration but the dye may not dry during the process since this causes disturbing fuchsin deposits.

Microscopical aspect: everything is stained red, mycobacteria, other bacteria as well as cells and all other elements.

5. Gently rinse the slide with running water.

6. Decolorise by flooding the slide with acid-alcohol solution. Everything, except the mycobacteria (= Acid-Fast Bacilli =AFB) will loose the red colour. When, after rinsing, the smear still shows visual red spots, unstain again until the red colour has disappeared. The mycobacteria can hardly be decolorised !

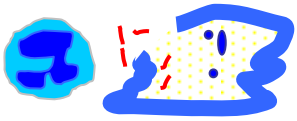


Microscopical aspect: all elements are decolorised*, except the mycobacteria.*

7. Gently rinse the slide with running water.

8. Flood the slide with the counter stain. The very fine, and generally rarely found, AFB are hardly visible and require a different background. Therefore, an optimal contrasting colour, methylene blue is chosen.

9. Let react for 2 minutes.



Microscopical aspect: all elements are stained blue, except the mycobacteria which remained red from the first staining action.

10. Gently rinse the slide with running water.

11. Wipe the back of each slide clean with a new tissue, and place them in a slide rack for the smears to air-dry but avoid direct sunlight.

12. Examine the slide under the microscope (immersion oil, objective 100x, eye piece 10x).

5. AURAMINE STAINING :

PRINCIPLE

The auramine stain is a relatively specific stain for Mycobacteria based on their resistance to decolorization by alcohol-acid after staining with a primary stain (auramine). This resistance is due to the presence of a lipid layer on their cell surface.

MATERIAL

Microscope slides, diamond-marking pen, wire loop or bamboo sticks, Forceps, Bunsen burner or spirit lamp, alcohol jar sand jar, filter paper, funnel, staining rack to hold smear slides, Auramine solution, alcohol-acid 0.5%, Loeffler methylene blue, LED microscope (objective 20x, 40x. [50x], excitation filter 450nm, emission filter 500nm), soft paper.

METHOD

1. Put the fixed slides on horizontal bars. Avoid contact between the slides in order to avoid cross contamination.
2. Cover the slide with filtered Auramine solution. DO NOT HEAT.
3. Let stain for 20 minutes. A longer time (> 30 minutes may produce artefacts

   Microscopical aspect: everything is stained yellow: mycobacteria, other bacteria as well as cells and all other elements.
4. Gently rinse the slide with running water.
5. Decolorize by flooding the slide with acid-alcohol 0.5% solution.
6. Allow to act for 3 minutes. Everything, except the mycobacteria (= Acid-Fast Bacilli =AFB) will lose the yellow fluorescence. If after rinsing, the smear still shows visual stains, repeat decolourisation. The mycobacteria can hardly be decolorized!

   Microscopical aspect: all elements are decolorized*, except the mycobacteria.*
7. Gently rinse the slide with running water.
8. Flood the slide with the Löffler methylene blue solution.
9. Let react for 1 minute.

   Microscopical aspect: all elements are stained blue, except the mycobacteria which remained yellow fluorescent from the first staining action.
10. Gently rinse the slide with running water.
11. Wipe the back of each slide clean with a new tissue, and place them in a slide rack for the smears to air-dry but avoid direct sunlight.
12. Examine the slide under the fluorescent microscope (objective 20x, eye piece 10x for scanning, and 40x (or 50x for confirmation).

TUBERCULOSIS [2]

INTRODUCTION :

The total number of tuberculosis (TB) cases in the world is increasing over the last decades: about 7.5 million persons in 1990 over 8.8 million in 1995, up to 10 million infected people in 2002, spread over the whole world. According to the WHO, between 2 and 3 million of deaths a year are due to tuberculosis.
The causes of the spread of tuberculosis are related to malnutrition, overpopulation and lack of health care infrastructures.
HIV infections have an important negative role in TB disease : 40 % of the 40 million HIV seropositive subjects are co-infected by tuberculosis, both of them accelerating and worsening the disease process.

CHARACTERISTICS

The responsible bacteria for the tubercle bacilli disease are the species *Mycobacterium tuberculosis* (the most virulent in human), *Mycobacterium Bovis, Mycobacterium bovis BCG (bacil of Calmette-Guerin)* and *Mycobacterium africanum*, *Mycobacterium microti and Mycobacterium canettii,* commonly called the Koch bacilli and referred to as the M. tuberculosis complex.

Belong to the genus ***Mycobacterium***, a group with about 70 described species most of which are saprophytic yet some of them can cause opportunistic infections in immunocompromised subjects (non typical mycobacteria : *Mycobacterium avium*, *Mycobacterium arkansi* ….)

All these bacteria:

- Have no morphological differences between the different mycobacteria species.
- Are **Gram positive**
- Are **acid resistant** in the Ziehl stain: the lipoid structure of the capsule of Mycobacteria makes them resistant to acid and alcohol, whereas all other bacteria aren't. There are a few exceptions (*Actinomyces, Nocardia, Corynebacterium* and the oöcysts of *Cryptosporidium)* but these organisms have a distinct shape.
- Are Fine, rod-shaped bacilli, 3-5 µm length, slightly arched, often with 2, sometimes in chains.
- Have **no spore** developing
- Are **immobile**
- Are **aerobe**
- Can mostly be **cultured** on special culture medium, Lowenstein-Jensen for example (slowly growing within 3 - 8 weeks).
- Are sensible for U.V. light
- Are rather resistant to hypochloric disinfectants but sensitive to Dettol.

LOCALITATIONS

TB generally affects **lungs**, but other organs can be also affected. In countries with low income and high tuberculosis prevalence, sputum smear microscopy is a cost effective tool for diagnosing patients with infectious tuberculosis.

SPECIFIC PRECAUTIONS FOR TUBERCULOSIS

Since tuberculosis is an airborne bacteria, transmitted by the spread of air micro particles of 2 to 10 µm, the collection and the preparation of sputum samples implies a potential risk. Safety measurements for health care workers (HCW) must therefore be considered: all precautions must be taken and rigidly applied to reduce **production of and contact with aerosols.**

[2] Resources internet utiles : http://www.who.int/gtb/publications/TBCatalogue.htm
http://www.iuatld.org/full_picture/en/frameset/frameset.phtml [for example in pdf format : TECHNICAL GUIDE Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries]

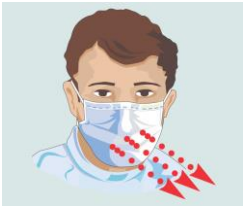
HCW should consider that handling tuberculosis samples involves some biohazard. All manipulations should be performed according to carefully described procedures with strict safety conditions.

Contrary to all other microorganisms, diluted hypochlorite (bleach) is not a good disinfectant for mycobacteria. Dettol 10 % is generally used instead.

The work in safety cabins (laminar flows) can only be considered if the instrument is submitted to a permanent quality control in order to guarantee its uninterrupted well-functioning. In all other circumstances it is really dangerous to handle tubercle samples in it, because of the risk of aerosol formation! A separate and well ventilated room should be used. Good ventilation will protect the laboratory staff from airborne infectious droplet nuclei. An easy way to ensure ventilation and directional airflow is by judiciously locating the bench, in function of windows and doors so that airborne particles are blown away from the laboratory worker. When electricity is available, extractor fans can be used to remove air from the laboratory.

The use of protection masks should be considered: masks of class P2 (FFP2) conform with the European standard (equivalent to the N95 American regulation), which permits filtration of drops bigger than 0,6 µm with an efficiency in the range of 80 % for P1, taking into account the possible adherence of spots at the face and leakage of the masks (persons with a beard....).
The correct use of the mask is mandatory for its efficiency: directions for use must be available and be instructed to all staff that needs to wear it. It has a limited time efficiency (most of the time 3 hours, depending on the manufacturer).
In view of the high price (minimum 0.50 € piece, mostly over 1 € in 2005) its use ought to be restricted: e.g. 1 mask per person per day, since it is rare to stay longer than 3 hours in a contagious area.
In zones with low risk of drug resistance and in well ventilated rooms, the wearing of masks should not be advised except maybe for lab technicians during sputum collection and during the preparation of the smears.

The wearing of an anti-inhalation mask does not protect a 100% and is only an expensive, complementary measurement to the total of safety precautions (sputum collecting outside in open air, being aware of the wind direction, ventilation, decontamination, large windows permitting to enter UV**,** reduction of working time in the hazardous zone, subdivision of the patient's zone, proper incineration of the biohazard waste, UVC lamps, etc.**). Improper use of these masks can have a wrong effect by only giving a feeling of protection.** The high cost of the masks might be a pretext to re-use them, which can be dangerous.
Surgical masks are not respirators, they do not offer protection to the person wearing the mask.

Breathing protections masks (FFP : Filtering Face piece Particles) : reduce inhalation of aerosols particles and air suspended drops. **→ Protection of the person wearing the mask against inhalation risks of biohazards.** 	**Medical masks:** obstruction of drops dispersed by breathing. **→ protection of persons in the proximity of the mask-wearing person against aerosols.** 

Performances of respiratory protection masks. Essays (particles of 0,01 to 1 µm) :

Type	Filter penetration [3]	Total leakage [4]
P1 or FFP1	< 20 %	< 22 %
P2 or FFP2	< 6 %	< 8 %
P3 or FFP3	< 0.05 %	< 2 %

[3] The disposable one-way-masks of type FP1 to FP3 protect against aerosols (suspensions of solid or liquid particles), but they protect by no means against vapours or gas.
[4]The global efficiency of a respiratory protection piece does not only depend on the efficiency of the filter, but also on the leakage between the filter and the face. It must be remarked that the efficiency of a mask is dramatically reduced by a beard (even after one or two days). This type of masks is therefore should be dissuaded for people with a beard.

Respirator Fitting instructions:
Respirators are effective when there is a good seal between the mask and face. When this seal is altered protection is compromised because the contaminated air can pass through the sides of the mask. Therefore respirators should not be used by people with facial hair and staff who use respirators must be trained.

	1B. Cup the respirator in your hand, headbands hanging freely
	2. Position the respirator.
	3. Pull the top strap over your head at position it high at the top back of your head. Pull the bottom strap over your head and position it around the neck below your ears. (Make sure the straps are not twisted)
	4. Place the fingertips of both hands at the top of the metal nosepiece. Mould the nosepiece to the shape of your nose by pushing inward while moving your fingertips down both sides of the nosepiece.
	5. The seal of the respirator should be checked prior to wearing in the work area (= Fit check)

Note:. Do not place the respirator under your chin or on your head when answering the phone or talking.

Fit checking:
A fit check assures that the respirator is sealed against the face. It should be done each time the respirator is worn.
-Positive pressure fit check:
Cover the entire surface of the respirator with your hands. Exhale sharply. If air is felt escaping around, readjust the noseclipor work the straps to eliminate leakage. Retest until the respirator fits properly.
Negative pressure fit check:
Again cover the respirator with your hands. Inhale, this should create a vacuum, causing the respirator to be drawn slightly toward the face. If not reposition and/or examine for defects (small hole). Retest until the respirator fits properly.

Removing a respirator:
Remove gloves and wash hands before removing respirators. Only the strap should be handled, avoid touching the front of the respirator and dispose with infectious waste.

Note: Not all respirators fit equally well to all persons. A fit test can help to choose the right brand/model and size but needs specialized equipment (ex. 3M Fittest FT10) and expertise on how to perform the test.

SAMPLING FOR PULMONARY TB

The risk of infection for health care workers is highest when TB suspects cough. Sputum specimen should therefore be collected in the open air and as far away as possible from other people (take care of the wind direction !). If this is not possible, a separate well ventilated room should be used.

- The health care worker should provide a sputum container with patient's identification written on the side of the container (never only on its lid)

- The best types of sputum containers are disposable with a water-tight screw cover to avoid leakage, and a useful (wide-mouthed) diameter of at least 35 mm and a volume capacity of 50 ml. These should be made of break-resistant plastic. It should be made of translucent material in order to allow specimen inspection without opening the container. The side should be easy to label in order to make a clear and permanent identification of the sample.
  The material must be single-use combustible to facilitate disposal. As a rule no recycled vials are used. If this is really impossible, the heavy glass 28 ml Universal bottle may be used, which can be re-used after thorough decontamination, cleaning and sterilisation

- The patient must be clearly instructed in advance of the sample procedure. During instructions, the HCW must stand aside the patient and not in front of the person. The expectoration must come from the depth and the subject must cough thoroughly to avoid that the sample should be too strongly contaminated by commensally oropharyngal bacteria. The presence of white, foamy saliva in this context is suggestive for oral contamination.

- The health care worker should make sure that the specimen is of sufficient volume (3 to 5 ml) and that it contains solid or purulent material. The sputum colour of TB patients can vary from yellow to brown and green and sometimes red from blood. A cheesy aspect is rather typical. Saliva is not suitable (lack of sensitivity). Sputum will not be always purulent, especially not during the treatment.

- The quality of the sputum may be checked after smearing and staining. It is accepted that there should be 20 times more WBC present in the sample than squamous epithelial cells.

- Two or three expectorations are collected over two or more days, the first during the consultation, the second at home and eventually a third one at the next visit, since lung lesions drain intermittently.

- An early morning specimen of sputum is preferred. These have the highest bacteria concentration and aren't contaminated with food particles.

- A sputum sample can be stored rather long (more than a week) in the refrigerator, for microscopical examination (of course culture will fail with long delays). In fact, homogenization of sputum, which occurs spontaneously after some days, may increase a little bit the sensitivity.

- For children, who are unable to provoke sputum, stomach tubing can be performed, in order to collect swallowed sputum during the night.

- Another method is sputum induction: the sputum is induced by letting the patient aspirate a hypertonic (3 – 5 %) salt solution, with a dispenser. This might realise a little increase of sensitivity, but with an increasing of risk of transmission (dispenser contamination, …).

SAMPLE CONCENTRATION METHODS

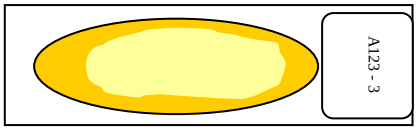
Several methods for concentration of TB exist: hypochloric solution, flotation… However they are rarely used, since these manipulations result in little sensitivity gain and contain a danger of aerosol formation.

THE SMEAR

The risk of contamination of laboratory personnel during the preparation of the smear is small but real (see precautions page 14-15). It is therefore recommended to prepare smears in a separate well-ventilated room (if possible, use an air extractor – kitchen hood type, and note the direction of air currents), and avoid all risks of aerosol production. Good ventilation will protect the laboratory staff from airborne infectious droplet nuclei. The use of a laminar flow is only advisable if the cabinet can be regularly and completely controlled. Consider wearing a mask FFP2 during smear preparation. During sample manipulation, vials and slides can be put on absorbent paper soaked in Dettol to 10% (decreased risk of aerosol production).

As a rule, **only new, clean, greaseless and unscratched microscope slides** are used for TB examination.

The identification (laboratory code, patient number, sample number) from the container is carefully taken over on the slide with a permanent (diamond-point or wax-pencil) marker.
Gently and carefully open the sputum container and verify the identification number on it.



With a broken wooden (bamboo) stick (or an inoculation loop) a consistent portion from the most solid and purulent part of the sputum (blood-specked, opaque, greyish or yellowish cheesy mucus) is taken. Use the broken end of the two pieces of the applicator to break up larger particles.
The sputum specimen must be smeared homogeneously in a thin layer so that through the dry smear a text can still be read. Avoid putting too thick sputum parts on the slide.
The smear is made elliptically with a length of ± 2 cm by 1 cm (this length correspondents with 100 fields at objective 100x).

If an inoculation loop is used, it must be flamed to red-hot after a mechanical decontamination in sand and disinfectant. Wooden sticks must be properly evacuated in the contaminated waste bin.
Gently and carefully close the sputum container and verify the identification number on it.
Let the smears dry in the air for at least 15-30 minutes.
Subsequently the smears are gently fixed in a flame (or in ethanol or methanol, which conserves better the morphology of the bacteria). Fixation permits a long delay before staining in order to organise a batched staining procedure.

MICROSCOPICAL DIAGNOSIS OF PULMONARY TUBERCULOSIS :

The exclusive acid resistant capacity of mycobacteria makes it possible to execute a specific staining. This staining can make the distinction between *Mycobacteria* (which are thus called “acid resistant rods or acid-fast Bacilli - AFB”) and all other bacteria (acid unfast bacteria). Only mycobacteria resist the decolorizing process by acid-alcohol and retain the primary arylmethan stain. Generally the red alkali fuchsin dye is used for it. (Alternatively the auramine stain can be used, which gives a yellow fluorescent colour, followed by a counter-staining in order to highlight the “acid-fast” bacteria). Microscopically no distinction can be

made between *Mycobacterium tuberculosis* and other mycobacteria. They all are acid resistant. The localisation and the degree of endemic spread might be although suggestive for a certain ethiology.

Note: Few other organisms are, however, also Alcoolo Acid-Resistant: Corynebacteria, Actinomyces, *Nocardia* spp, *Cryptosporidium* oocysts. ...

Two microscopic systems are useful for detecting the presence of AFB

- **Light optical microscopy**: with ziehl-neelsen stain stays, after all, the most convenient technique in the field, thanks to the very short time needed for obtaining results, the restricted equipment, and a satisfying high sensitivity. Although well trained and highly motivated laboratory technicians must perform the examinations with a great sense of accuracy and responsibility.

  A good, well-maintained microscope, by preference a binocular one, is required for this purpose:
  - If small quantities of samples have to be examined, a monocular microscope can be used as well, moreover they have a stronger light intensity.
  - The light can be natural or any other source of light.
  - A good mechanical stage is needed for a good screening

  The examination is done with ocular 10 x, objective 100 x and immersion oil

- **Fluorescence microscopy** : with auramine stain. This method is quicker than visual light microscopy (obj. 20 x is used) and more sensible, but the specificity is lower and by that the positive samples must be confirmed by normal light optical microscopy, at least at the beginning. It is more expensive, it needs electricity and is technically more demanding. This technique is advised for bigger laboratories with a higher examination capacity (more than 50 (30 ?) samples per day).

  New development of LED light sources adjusted to fluorescence microscope may overcome the technical difficulties. LED fluorescent microscope are less complicated, less expensive and more stables than the normal fluorescent microscope. (Example: microscope fluoLED Easy Royal Blue for Olympus CX 21 from FRAEN : +/- 3.000€). Another advantage in comparison to the normal fluorescent microscope is that they can be used outside an “dark room”.

Comparative table between normal and fluorescent microscope:

Traditional microscope ZIEHL-NEELSEN technique	LED fluorescent microscope Auramine technique
• Microscope: not expensive and polyvalent.	• Microscope: expensive and preferably used only for fluorescence
• Very stable reagents (long expiration, not temperature sensitive).	• Less stable reagents (short expiration, temperature sensitive: if > 40°c) ➔ Logistical problem for the distribution of the reagents).
• Low sensitivity.	• Better sensitivity principally due to the shorted reading time needed.
• Quality control possible without re-staining during some months.	• Quality control possible without re-staining during 1 month.

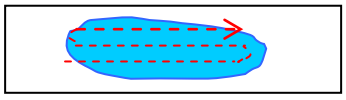
N.B.: If the both techniques are in use in a health system, there is a risk that the highest level (using the fluorescent microscope) will lose the capacity to supervise the lowest level (using the traditional microscope).

Numerous methods have been developed for acid-fast microscopy (Ziehl-Neelsen, Kinyoun, Tan Thiam Hok methods, …using the same reagents but with different concentrations or with different incubation times or at a different temperature). There even exists a method for colour-blind people, the Tison technique, which uses different products, which stains the AFR blue on a yellow background. All of these have some advantages and some disadvantages. Standardization of the methods throughout the country is more important than which method is chosen.
Only the hot Ziehl-Neelsen procedure will be explained here. This technique is often preferred to the cold staining. The fuchsin is heated underneath of the preparation, with a small alcohol flame, until vapour just begins to rise, but do not overheat. This might result in a better staining, although this method is much more labour intensive. (Each staining must be performed individually). It also releases toxic vapours, more staining solution is used and there is a risk of burning and degenerating of the smear material.

MICROSCOPICAL EXAMINATION OF THE PREPARATION

A microscope technician should not do more than 25 preparations per day since the work is very tiring and asks a permanent attention. But the workload should be high enough to maintain testing proficiency (at least 1 positive sample per week).

Systematically search during 5 - 10 minutes or 100 overlapping fields. It is important to use the fine adjustment knob to observe possible presence of AFB in the entire thickness of the preparation. A prolonged examination delivers no substantial gain of sensitivity. According to estimations it takes about 50.000 to 100.000 AFB/ml (10.000 AFB/ml ?) sputum to find a smear positive. Mycobacteria are found mainly in cellular and mucosal parts of the preparation. Typical AFB are thin, often curved sticks stained deep red. They are either isolated or grouped in clusters or strings.



SEMI QUANTIFICATION SCALE

Positive smears need to be quantified, for proficiency testing and also since this helps in interpretation, especially for follow-up smears. The results are expressed in a semi quantitative way as e.g. scale of the WHO or the scale of the American Thoracic Society (Lennette**)**

Number of AFB per fields	WHO/UICTMR scale	ATS scale	Minimal number of fields counted
Empty	Negative	Negative	100
Less than 1 AFB/ 100 fields	+/-	+/-	200-300
1 – 9 AFB / 100 fields	+/-	1+	200-300
1 – 9 AFB / 10 fields	1+	2+	100
1 – 9 AFB / 1 field	2+	3+	50
10 AFB per field	3+	4+	20

The difference between the WHO and the ATS scale is the choice of the threshold: 1 AFB/10 fields. The first will result in a higher specificity, implying a lower sensitivity.

It is best to use the quantification scale recommended by the national program in the country. The microscopist must report what he saw, without interpretation. This means reporting AFB and not the diagnosis of tuberculosis. If the number of AFB seen finds himself at the threshold of the scale value (+ / -), the result must remain the number of AFB and not be turned into a positive or negative.

Rough equivalent of result between different staining techniques (fuchsin vs. auramine)

ATS scale [5]	Number of AFB per fields (Ziehl-Neelsen) (1.000x magnification)	Number of AFB per fields (Auramine) (200x magnification)
Negative	Zero AFB / 100 fields	Zero AFB / 100 fields
+/-	Less than 1 AFB/ 100 fields	Less than 1 AFB/ 100 fields
1+	1 – 9 AFB / 100 fields	1 –29 AFB / 100 fields
2+	1 – 9 AFB / 10 fields	1 – 9 AFB / 1 fields
3+	1 – 9 AFB / 1 field	10 – 99 AFB / 1 field
4+	10 AFB or more per field	100 AFB or more per field

In the examination report, only presence (+ quantification) or absence of AFB is mentioned. All the other bacteria are not considered.

INTERPRETATION OF RESULTS

- The sensitivity of microscopy for pulmonary tuberculosis depends on different factors; stage of the disease, associated immunosuppression, severity of symptoms, quality of sputum, quality of staining solutions and dyes, quality and condition of the microscope, motivation and experience of the microscopist. The sensitivity of microscopy for pulmonary tuberculosis is estimated around 75 %. This sensitivity is lower for children (difficulty to obtain sputum) and for immunocompromised patient (around 60 % or less).

- The detection rate, in function of the number of sputum examined is about :
  77 - 95 % after 1 examination
  \+ 4 - 18 % after the second examination
  \+ 1 - 3 % after the third examination

[5] **ATS :** American Thoracic Society.

- The specificity is 97 %. (saprophyte mycobacteria,…)
- To initiate treatment, you must have at least two positive samples (+ / -).
- If the examination is negative for the 2 or 3 sputa, the entire procedure is repeated on new sputum samples (two weeks after), and if still negative, a third time after a month. Further investigation does not contribute to a better sensitivity.
- Ziehl-Neelsen on sputum can also be used to follow-up effectiveness of treatment: at the end of the intensive phase of treatment, the 5th month and end of treatment, for example. Reading these slides is more difficult ("dead" granular bacteria). The ideal technique to follow-up treatment effectiveness would be the culture, however this is not possible in small laboratories.

PROFICIENCY TESTING OF THE SPUTUM SMEAR MICROSCOPY

It should be stressed that acid-fast microscopy is not difficult to learn; its challenge lies with the sustainability of continued high quality.
The purpose of a quality assurance program is the improvement of the efficiency and the reliability of smear microscopy services.
If feasible, an internal quality control should be done on daily basis.

METHODS :

All methods have distinct advantages and disadvantages. It is thus advisable to develop several methods in parallel.

- Supervision : visits to the peripheral laboratories by **exper**ienced staff (from national or intermediate laboratories)..
  Advantages : Direct contact ➔ Motivating to staff.
  Identifies causes of errors ➔ correction on the spot.
  Permits verification of the quality of equipment and stocks.
  BUT : Costly and labour intensive ➔ complementary to other methods.

- Quality control :
  By "Panel tests" : Known slides are sent, without results, to peripheral laboratories. All peripheral laboratories have to return the results to the central laboratory. The advantage of this method is the ability to obtain a quick assessment of the technical ability to read smears in a target area with very little effort from both reference and peripheral laboratories. The major disadvantage of this method lies in the fact that technicians have an unlimited time for the examination of control slides and they are aware of being tested. Therefore, this method does not allow the assessment of the quality of slide reading under routine conditions. This system tests only the **capacity** of a laboratory.

  Control of the routine slides by a reference laboratory .This is the best system since it is motivating and gives an immediate and realistic idea of the work. This system tests the **performance** of a laboratory.

PRINCIPLES (for re-examining a sample of routine slides) :

- As small as possible quantities are controlled.
- Statistical and technical reliability must be targeted.
- Positive samples ? : Shouldn't cause any problem or there may by systematic failures ?
- Negative samples ? : Normal errors, critical values may not be exceeded?

AIM:

- To get an idea of the performance level of the service.
- To detect centres that may have an unacceptable level.
- Quality improvement and **not** diagnosis correction or individual microscopist evaluation.

ATTENTION ITEMS FOR CONTROL :

- Samples must be chosen random and representative:
- A choice out of **all** examined samples must be possible.
- The proportion of positives, rare mycobacteria and negatives must be the same as in the daily practice.
- The first reading is blind; this also brings along mistakes.
- A second reading is only done for discordant results.
- Re-staining:
  - Best before the first re-reading: for detection of bad staining.
  - Surely before the second re-reading, since fuchsin fades out.
- Validation of results:
  - Failure percentage of the first supervisor versus the controlled person is sent back, along with the slides with serious mistakes.
- Feedback with identification of the causes of errors, followed by corrections!

CAUSES OF ERRORS

FALSE POSITIVES	**FALSE NEGATIVES**
❑ Administrative errors, mix-up of samples ❑ Too strong decentralisation, lack of expertise ❑ Recycling of used slides! ❑ Scratches ❑ Artefacts ❑ Alimentary particles ❑ Dye deposits ❑ Spores, fibres, pollen ❑ Other bacteria or mycobacteria (saprophytes in tap water) ❑ Contamination ▪ from another sputum ▪ from dye-flasks ▪ from filter paper or absorbent paper ▪ immersion oil from the oil dispenser ▪ dirty objective! This should be thoroughly wiped after each lecture of slide**, …** ❑ … ➔Too strong decentralisation, lack of experience, lack of expertise, lack of financial support, …	❑ Bad sample (saliva) ❑ Bad dyes ❑ Bad staining technique ❑ Bad quality of microscope ❑ Insufficient lecture of the smears ❑ Negligent microscopy ❑ Lack of motivation ❑ Overcharged staff. ❑ Too thick smears ❑ Insufficient light ❑ … ➔ Workload, motivation, microscope quality, …
	NONSENSE RESULTS
	❑ Administration ❑ Complete lack of training ❑ Totally useless microscope ❑ …

NON TECHNICAL CAUSES OF ERRORS

- ❑ **M O T I V A T I O N**
- ❑ Bad lab organisation
- ❑ Workload
- ❑ Forgery of results (to obtain medication, free treatment…)
- ❑ Fear of a positive result
- ❑ …

MICROSCOPICAL DIAGNOSIS FOR EXTRA-PULMONARY SPECIMEN

Direct examination of specimen other than sputa has a very low sensitivity (although most of the time a high specificity). When found negative one should examine these samples with other methods (culture…) or send it to a specialised laboratory.

- Spinal fluid: the concentration of TB is often low. Therefore, it is possible to concentrate the specimen by centrifugation (20 minutes, high speed). Low sensitivity (< 20 %), but high specificity.

- Gland: do not puncture the gland because of danger of fistula formation, but make a dissection. Cut the gland into two parts and with one half make prints on a slide = deps preparation. (Sensitivity ± 35 %, high specificity).

- For aspirates as from pleura, pericardium and peritoneum, the Rivalta test can be done, together with a cell differentiation and a Ziehl staining. (Sensitivity for AFB ± 5 %, high specificity)

For some samples, the direct examination will not help a lot :

- Kidney tuberculosis: examination of morning urine after high speed centrifugation, but this investigation has a low sensitivity and also a low specificity by the presence of non tubercle mycobacteria.

- Pus and thick aspirates: make very thin smears since thick smears tend to float of the slide. Examination is difficult because acid-fast bacilli are often hidden and blood may sometimes produce acid-fast artefacts.

EXAMINATION OF PUNCTURE FLUID:

PRINCIPLE :

A serous puncture does not generate fluid in healthy persons, only in some pathologies fluid is produced. Analysis of this bodily fluid (evaluation of proteins and differentiation of cells) offers a good diagnostic orientation.

SAMPLING :

Puncture fluid (ascites fluid or pleural fluid) is collected in a syringe or in a clean and dry tube.

THE RIVALTA REACTION IS NOT INTERPRETABLE IN PRESENCE OF BLOOD, BECAUSE OF CONTAMINATION BY BLOOD PROTEINS (HAEMOGLOBIN AND PLASMA)

RIVALTA REACTION :

PRINCIPLE :

The Rivalta reaction is proving the presence of proteins in a liquid by precipitation in an acid medium. This test allows differentiation of liquids with high concentrations of proteins (> 30 g/l) from low concentrated liquids (< 20 g/l). It is only applicable on puncture fluids (in function of the concentration of the expected protein values).

MATERIAL :

Graduated cylinder of 100 ml, distilled water (or pure or filtered water), Pasteur pipettes, glacial acetic acid, black cardboard.

METHOD:

1. Measure 100 ml of water (distilled or filtered) in a glass graduated cylinder.
2. Add, with a Pasteur pipette, 3 drops of glacial acetic acid (100 %) and mix this solution.
3. Pour gently with another Pasteur pipette, a drop of the fluid to analyse on the surface of the solution.
4. Observe the diffusion of the drop of the fluid in the solution.
5. If the drop is immediately and completely dissolving in the solution, the reaction is **negative.** The fluid has a low protein concentration and is called "***TRANSSUDATE"***.
6. If the drop is forming a smoky precipitation (cigarette smoke alike) or if the drop is coagulating strongly, the reaction is **positive.** The fluid has a high protein concentration and is called ***"EXSUDATE"***.

ANSWER TYPES :

Macroscopic aspect of the puncture fluid :

- Rivalta positive : EXSUDATE.
- Rivalta negative : TRANSSUDATE.

CELL DIFFERENTIATION:

PRINCIPLE :

The centrifuged sediment of the fluid is stained and microscopically examined. The leukocytes differentiation permits a diagnostic orientation.

MATERIAL :

Centrifuge, centrifugation tubes, slides, diamond marker, sand/cresyl mixture, Giemsa, buffer water, [May-Grünwald], timer, wire loop, alcohol lamp, (methanol), slides staining support, slides dry support, microscope, immersion oil.

METHOD :

1. The liquid specimen is transferred into a conical centrifugation tube and centrifuged for 10 minutes at 1.000 g.

2. Pour off gently the supernatant. Spread out a drop of the sediment on a slide.

3. After drying, the slide is fixated with methanol, followed by Giemsa staining as for a thick smear.

4. Read the slide under the microscope with objective 100 x. Evaluate the number leukocytes, subsequently differentiate as for a leukocyte formula. **Take care, the leukocytes are often deformed. On should not confuse them with endothelial or neoplastic cells.**

ANSWER TYPES :

- Estimation of the number of leucocytes : Rare, small amounts, numerous cells, very numerous cells.

- Neutrophils percentage: _ _ _ %
  Eosinophils percentage: _ _ _ %
  Lymphocytes percentage : _ _ _ %

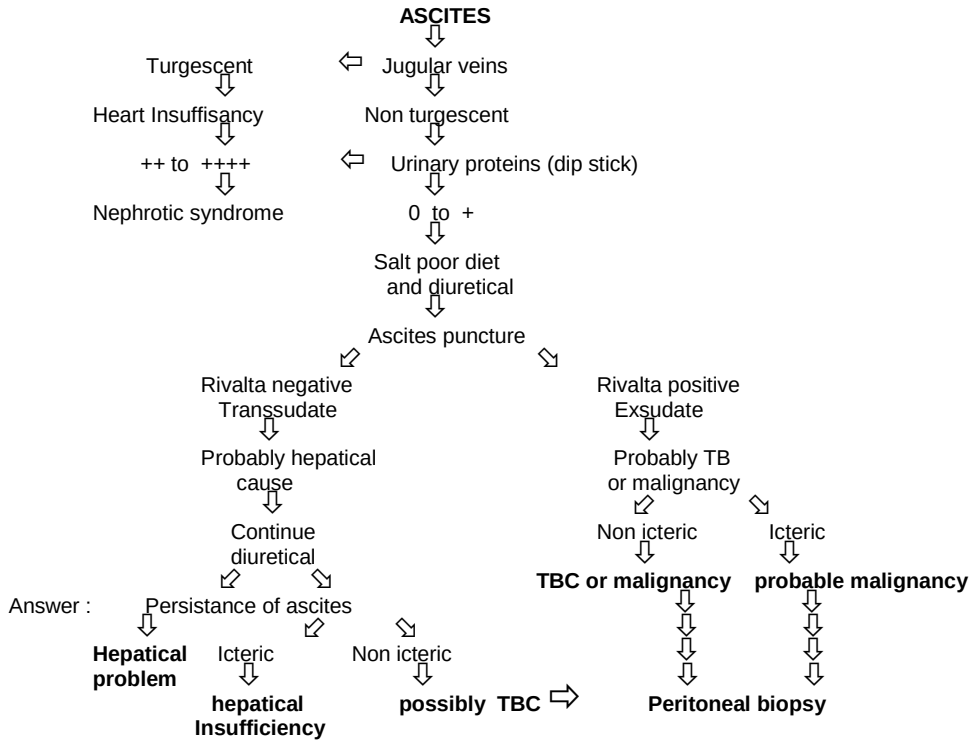
INTERPRETATION OF THE RESULTS :

MACROSCOPICAL ASPECT OF THE PUNCTURE LIQUID	WHAT IS PRODUCED
Serous : clear, opalescent	Transsudate
Sero-fibrinal : lemon yellow, amber	Exsudate
Festering : thick, yellow greenish or brown	Pleural ulcer
Haemorrhagic : red or pink	Accidental vein stick Traumatism, Tuberculosis or neoplasy
Milkish, whitish.	Lymphatic filariosis
Rocky water : limpid and clear.	Hydatidosis (rare)[6]

FLUID FROM PLEURAL PUNCTION		
ORIGINE	**APPEARENCE**	**TYPE OF CELLS**
TRANSSUDATES (Rivalta negative)		
Cardiac or renal	Clear	Few cells, lymphocytes
EXSUDATES (Rivalta positive)		
Bacterial pleurisy	Festering	Very numerous neutrophils
Tuberculosis	Clear	Numerous lymphocytes
Cancer	Haemorrhagic [7]	Red blood cells
ASCITES PUNCTURE FLUID [8]		
ORIGINE	**APPEARENCE**	**TYPE DE CELLULES**
TRANSSUDATES (Rivalta negative)		
Cirrhosis	Yellow	Low number of neutrophils
Cardiac	Yellow	Numerous mononuclear cells
Renal	Yellow or whitish	Absent
EXSUDATES (Rivalta positive)		
Tuberculosis	Clear, trouble, or whitish, sometimes haemorrhagic [9]	Numerous lymphocytes over 70 % [9]
Cancer	Yellow or whitish, sometimes haemorrhagic [9]	Variable, numerous

[6] The puncture of an hydatic cysts is strongly discouraged because of the high risk of dissemination.
[7] The Rivalta is always positive on haemorrhagic fluids since this contains haemoglobin and blood plasma both composed of proteins.
[8] This table includes about 90 % of the ascites origins.
[9] The Zhiehl stain will be positive in less than 5 %.

DIAGNOSTIC STRATEGY EXAMPLE FOR ASCITES (ACCORDING TO P.A. REEVE)[10]



BURULI ULCER[11]

INTRODUCTION:

Buruli ulcer, a rising disease in full expansion, is the third most spread mycobacterial infection after tuberculosis and leprosy. The causal bacteria are *Mycobacterium ulcerans* and provoke serious ulcerations of skin, muscles and bones leaving its victims gravely malformed or even enabled.

The WHO considers Buruli ulcer as a rising public health threat. This disease is prevalent in about thirty countries: the zones swampy tropical and subtropical regions of Africa, Latin America, Asia and the West Pacific. It is especially spread in West Africa, where the dissemination of the infectious agent seems to accelerate since 1980. In Ghana, for instance, 22% of village people are infected in some regions and 16% of the population of some villages of Ivory Coast is infected.

In endemic zones, the diagnosis is mainly clinically based. In a low budget laboratory it is although possible to realise a Ziehl-Neelsen stain from a swamp specimen collected from the detached border of a not (or little) painful ulcer. The sensitivity of this technique however is not very high but other techniques, such as culture, histology or PCR cannot be considered in resource poor settings.

[10] Ascites : a guide to diagnosis in the district hospital. P.A. Reeve. Tropical Doctor (1992), 22, 52 – 56.
[11] Manual "Buruli ulcer, diagnosis of mycobacterium ulcerans disease" available in pdf format: http://www.who.int/gtb-buruli/publications/PDF/BURULI-diagnosis.pdf

LEPROSY

INTRODUCTION :

Leprosy is an infectious transmissible disease, caused by *Mycobacterium leprae* or bacillus of Hansen (1873). *Mycobacterium leprae* belongs to the genus Mycobacteria are alcohol-acid resistant rods of 1 to 8 µm length and 0.3 – 0.5 µm width. The bacteria develop intracellular, cannot be cultured in vitro but can be inoculated in the mouse foot sole and in the armadillo. Since 1985, the prevalence declined worldwide with 90 % and more than 13 million of leprous persons are cured with multichemotherapy. In 2004, the WHO considered leprosy no longer as a problem of public health (prevalence rate superior to 1 case on 10.000) except for 10 of 122 initially hit countries: Angola, Brazil, Congo, India, Liberia, Madagascar, Mozambique, Nepal, Central African Republic and Tanzania. In 2003 these countries where responsible for more than 86 % of the world prevalence and for 88.5 % of the new detected cases in 2002. At the beginning of 2002, 650.000 cases registered were under treatment.

DIAGNOSIS OF LEPROSIS :

The definition of the WHO of leprosy is « a sick person presenting signs provoked by leprosy, with or without bacterial confirmation and who needs a specific treatment». The disease is called paucibacillary leprosy (PB) when an infected person shows 1 to 5 cutaneous lesions, and multibacillary leprosy (MB) when a subject presents more than 5 cutaneous lesions (1998). For the treatment, pauci bacillary leprosy is still subdivided in leprosy with one lesion and leprosy with 2 to 5 lesions. The bacilloscopy is no longer indispensable for making a therapy decision. It is more and more given up in the field. The evaluation of a leprosy infection and the modalities of the immunological response are no longer realised (classification according to Ridley and Jopling in undetermined, tuberculoid, borderline, lepromatous), Intra Dermo Reaction, using the lepromin (reaction of Mitsuda) is no longer used in routine. The clinical classification in PB with one lesion or multiple and MB is sufficient for starting up an adapted polychimiotherapy (PCT).

A serodiagnosis permits also to detect the specific surface antigens (PGL1) of the bacilli of Hansen even before appearance of clinical signs. However, unfortunately, the cost price makes it only accessible for industrialised countries.

The specific antibody detection (anti-PGL1) can also be used. Its level is proportionally related to the bacillary charge. The sensitivity of this test for patients in the tuberculoid stage is nevertheless only in the range of 50 %.

The PCR, realised on nasal secretions delivers unsatisfactory results (false positives: asymptomatic carriers).

The bacteriological examination of *M. leprae* is executed on bloodless scarifications of the earlobes (the bacilli of Hansen is multiplying the best at a temperature of 33°C) and on cutaneous lesions (at right angles on the interior growing zone of the lesion). The slides are fixated with formaldehyde or methanol and subsequently Ziehl-Neelsen stained [the decolouration is preferably done with acid alcohol of 1 %] and examined under the microscope with immersion oil. This examination permits the evaluation of the bacteriological and morphological index.
The bacteriological index (BI) expresses by a scale from 1 to 6 (scale of Ridley) the number of bacilli present in the lesion.
The morphological index (MI) is a delicate determination and expresses the percentage of the solid bacilli (homogeneously stained and morphologically intact) on the total number of bacilli (solids and non-solids). The solid bacilli were alive at the moment of their fixation on the slide while the non-solids where death.

QUANTIFICATION OF THE RESULTS :

Number of AFR per x microscopical	Scale of Ridley	Classification of Ridley and Jopling [12]	Minimal number of microscopical fields to observe
Absents	0	TT / BT **(PB)**	100
1 to 9 AFR per 100 fields	1 +	BT **(MB)**	100
1 to 9 AFR per 10 fields	2 +	BT / BB **(MB)**	100
1 to 9 AFR per 1 field	3 +	BB **(MB)**	50
10 to 99 AFR per 1 field	4 +	BB / BL **(MB)**	50
100 to 900 AFR per 1 field	5 +	BL / LL **(MB)**	20
More than 1.000 AFR per field	6 +	LL **(MB)**	20

INTERPRETATION OF THE RESULTS:

The WHO recommended strategy in the fight against leprosy is a mass strategy and therefore easy diagnostic tests are required. The choice of treatment depends on the classification in groups of pauci- or multibacillary forms. This method carries certain strategical advantages. The classification based on the number of lesions involves however about 2 % of treatment errors for multibacillary subjects who are treated as paucibacillary and about 20 % for PB persons improperly treated as MB. The first group of MB is at risk for relapse after PB treatment, while the other group doesn't show any important inconveniency by prolonged over treatment.

[12] TT : tuberculoid leprosy, BT : borderline tuberculoid leprosy, BB : borderline leprosy, BL : borderline lepromateuse leprosy, LL : lepromateuse leprosy.

MENINGITIS

INTRODUCTION :

Out of epidemic periods, the WHO estimates the number of yearly bacterial meningitis cases in the world on more than 1.2 million. These meningitis cases are assumed to be responsible for more than 135.000 deaths a year, with moreover a very important morbidity.

Beyond the epidemics, the clinical diagnosis of bacterial meningitis is difficult. A differential diagnosis is required between a bacterial (meningococci, pneumococci, *Haemophilus*, mycobacteria,…) and other causes, such as fungal (cryptococcose, candidose), viral (enterovirus, mumps virus, arbovirus, …), or parasitic agents (malaria, trypanosomiasis, toxoplasmosis, amoebae,…). The examination of the (cerebro)spinal fluid (CSF) is an essential step in the diagnosis. This analysis is based on macroscopical aspect, the measuring of the increase of leucocytes number and of globulins and, or, the detection of the bacteria or parasite that is causing the meningitis. Bacterial culture is the best method (golden standard) although difficult to realise on the field and causes a time delay.

At the onset of a meningococcal epidemic, it is important to confirm the aetiology of the disease, to enable the setting up of the appropriate measurements for prevention and treatment. In this context, rapid antigen detection tests might be useful (supplying also a minimal determination of serogroups). Nevertheless, for obtaining a reliable identification of the serogroup(s), or sub-type(s), and clone(s), about 25 spinal fluid samples must be collected for and shipped to a specialised reference laboratory (p.i. Statens Institute for Folkehelse [SIF], Geitmyrsveien 75, 0462 Oslo 4, Norway Fax : + 47 22.04.25.18). Each reference laboratory demands a different transport medium (Trans-Isolate [TI] for SIF). These 25 samples will also enable the determination of the sensitivity of the bacteria to antibiotics.

SAMPLING :

Cerebrospinal fluid is normally a sterile liquid. Each bacterium that is retrieved during the examination might be of enormous importance as a potential cause of meningitis. Therefor it is very important to work in aseptical conditions as much as possible. Between 5 and 10 ml of spinal fluid is collected in two sterile tubes.

Maximal conservation of the CSF, depending on the analysis to be executed (Since cells, trypanosomes and glucose in spinal fluid degenerate quickly, it should be recommended to be examined preferably immediately after sampling):

Conservation	**Cytology** (Giemsa) **And staining** (Gram and Bleu)	**Detection of antigens** (latex)	**Culture or Inoculation Trans-Isolate (TI)**	**PCR**
Time delay	Maximum 8 hours	48 hours [A few weeks]	1 hour	A few weeks
Temperature	Room temperature	2°C – 8° C [frozen]	Room temperature **Never refrigerate**	frozen

MACROSCOPICAL EXAMINATION:

The macroscopic aspect of the fluid is important for the choice of the analysis to be executed on the sample. The visual aspect can be:

- Festering or purulent
- Turbid or cloudy
- Clear and colourless
- Reddish and eventually slightly turbid
- Yellow (Xanthochrome)

Recapitulation table for examinations to be executed depending on the macroscopic aspect of the spinal fluid

<table>
<tr><th colspan="5">EXAMINATIONS TO BE EXECUTED DEPENDING ON THE MACROSCOPIC ASPECT OF THE CSF</th></tr>
<tr><th>RED</th><th>PURULENT</th><th colspan="2">TURBID or CLOUDY</th><th colspan="2">CLEAR</th></tr>
<tr><td>⇩</td><td>⇩</td><td colspan="2">⇩</td><td colspan="2">⇩</td></tr>
<tr><td>1 Centrifugation</td><td>1. Methylene blue
2. Gram</td><td colspan="2">1. Cell count
2. Centrifugation
3. Giemsa
⇙ ⇘</td><td colspan="2">1. Cell count
2. Pandy
⇙ ⇘</td></tr>
<tr><td></td><td></td><td>Neutrophils</td><td>Lymphocytes</td><td>≥ 5 WBC</td><td>< 5 WBC</td></tr>
<tr><td>➔ Pink supernatant + clot of non coagulated RBC :

Haemorrhagic meninx

➔ Colourless supernatant:

Blood contamination
Request new sample if possible</td><td>If no bacteria are found, consider the fluid as if it were cloudy.</td><td>4. Methylene blue
5. Gram
6. Fresh smear
7. Indian ink
8. (Serology syphilis : VDRL)</td><td>4. (Ziehl)
5. fresh smear
6. Indian ink
7. Methylene blue
8. Gram</td><td>3. Centrifugation
4. Fresh smear
5. Indian ink
6. Giemsa
7. Methylene blue
8. Gram
9. (Ziehl)</td><td>3. Centrifugation
4. (Indian ink?)
5. (Fresh smear?)</td></tr>
</table>

A yellowish fluid is suggestive for an old haemorrhage, for a severe jaundice or for a squeeze of the spine. In case of a squeeze of the spine, the liquid will coagulate in one mass within 10 minutes.

A cloudy, pink or reddish liquid usually indicates either a subarachnoidal haemorrhage (both liquid tubes have the same colour, and after centrifugation, the supernatant is pink), or an accidental puncture of a blood vessel (the second liquid tube is less coloured, and after centrifugation, the supernatant is colourless). It is very difficult to obtain correct results on such liquids (WBC counting is impossible in this case). A new lumbar puncture should be requested.

MICROSCOPICAL EXAMINATION:

A purulent liquid generally indicates acute bacterial meningitis. A Gram and methylene blue stain are performed for bacterial identification. If the bacterial etiology is proven, it is unnecessary to perform cytological and biochemical analysis. In case of negative bacteriological determination, the sample is treated further as if it were cloudy.

A cloudy liquid can reveal several etiological causes. Perform a cell count [dilute the spinal fluid ½ with Turk[13] solution, count cells in 9 big squares of 0.1 mm² in a Neubauer counting chamber, counting twice the leucocytes in the last square. The number of WBC must be multiplied by 2 (dilution factor) in order to obtain the number of cells per mm³ of the spinal fluid. A spinal fluid is considered turbid starting from 200 cells per mm³.
Subsequently centrifuge the liquid [10 minutes at 1.000 g]. A Giemsa stain is done on the centrifugation clot to determine the cell type (lymphocytes, monocytes, polynuclear cells). According to the retrieved cell type, either a Gram stain and methylene blue stain (neutrophils), or a Ziehl-Neelsen (lymphocytes) is performed on the centrifugation clot.
The Pandy test and the determination of glycorrhachia, executed on the supernatant, can be useful.
Consider as well trypanosomiasis (fresh smear) or a fungal infection (examination with Indian ink).

A clear liquid is much more difficult : First it must be determined whether there is a meningitis or not : Cell count [count without dilution of the spinal fluid] can give exclusion for this question. In case of meningitis (cell count superior to 5 WBC per mm³), it will be probably viral meningo-encephalitis. But in the onset of some forms of bacterial meningitis, the liquid can be perfectly clear. A beginning or decapitated bacterial meningitis, trypanosomiasis or a fungal origin must although be considered. The Pandy test and the determination of glycorrhachia can give support.
Other infectious diseases can also be found in a meningitis with a clear CSF showing predominantly lymphocytes : spirochetes : syphilis, leptospirosis, borreliosis; brucellosis; trypanosomiasis, … Serology can be done for syphilis and for leptospirosis.

P.S. : The use of urinary dip sticks (type Multistix 8SG of Bayer and ECUR Boehringer) on CSF has been studied for the diagnostic approach of meningitis, based on the level of glucose, proteins and the leucocytes. For turbid fluids (which can already macroscopically define a meningitis) results are relatively good, but for clear CSF the sensitivity is in the range of 25 %, what means that 3 on 4 of proved bacterial meningitis (by culture or other tests) of the clear fluids are not revealed as meningitis with urinary sticks. … Another approach has been performed with urinary sticks for urinary nitrites dosage[14] with similar results. **They cannot replace the laboratory techniques.** In field conditions without laboratory facilities, the clinical examination must be the base of diagnosis:

[13] In the context of suspicion of trypanosomiase, the dilution of CSF with Türck solution is not recommanded (lyse of trypanosomes by the Türck solution).
[14] Rapid diagnosis of bacterial meningitis using nitrile patch testing. C. Maclennan, E. Banda, E.M. Molyneux, D.A. Green. Tropical Doctor (2004) 34, 231 – 232.

GERMS WHICH CAN BE RETRIEVED IN CSF				
Form	**Morphology, Disposition and Localisation**	**Staining**	**Design**	**Probable Aetiology**
1 Cocci	Often rare, with 2, coffee been shape, intra- or extra-cellular.	Gram Negative		*Neisseria meningitidis*
2 Rods	Often rare, fine rods, coccoid, short chains, intra or extra-cellular, often encapsulated.	Gram Negative		*Haemophilus influenzae*
3 Cocci	Often abundant, lancet shape, with 2 or in short chains, often encapsulated., always extra-cellular	Gram Positive		*Streptococcus pneumoniae*
4 Big round elements	Big round elements with the size of a RBC often with button forming, sometimes with pseudomycelium [With capsule for *Cryptococcus neoformans]*	Gram Positive Indian ink		Yeasts *Cryptococcus neoformans*
5 Cocci	Regular cocci in groups, extra-cellular	Gram Positive		Staphylococci (*S. aureus*, *S. epidermidis*,…)
6 Rods	Small isolated rods, parallel in V form, in palisades, intra or extra-cellular	Gram Positive		*Listeria monocytogenes*
7 Cocci	Cocci with 2 or in rather long chains	Gram Positive		*Streptococcus agalactiae*
8 Rods	Big rods, often bipolar	Gram Negative		Enterobacteria *(Escherichia coli)*
9 Rods	Fine rods	Ziehl AFB		*Mycobacterium tuberculosis*

GRAM POSITIVE = DEEP VIOLET / BLUE
GRAM NEGATIVE = RED / PINK

The bacteria 1 to 3 represent 80 to 90 % of the bacterial meningitis. Found in all ages.
Neisseria species are responsible of most of the epidemics
The germ 2 is usually found in children of 3 months to 3 years.
The groups 4 and 5 are only found in immunocompromised subjects (or in case of foreign body [drain]).
Group 5 can be retrieved in persons older than 50.
The germs 6 to 8 cause principally meningitis in infants of less than 6 months.
Group 9 is microscopically demonstrated in less than 5 % of the tuberculosis meningitis.
Occasionally, each bacteria can cause a meningitis (*Pseudomonas spp.*, *Klebsiella spp.*, …)

ANSWER TYPES :

Aspect of the CSF :
WBC count : _ _ _ / mm^3 de L.C.R.
WBC differentiation : _ _ _ % Lymphocytes
_ _ _ % Neutrophils
Pandy :
Result of the Gram stain :
Result of the methylene blue stain :
Result of the Ziehl stain:
Result of the fresh smear :
Result of the Indian ink stain :

REFERENCES VALUES:

A normal CSF:

- Has a normal pressure (is dripping drop by drop from the syringe).
- Is clear.
- Has a proteinorachia between 10 and 45 mg% (different values according to the applied measure method).
- Is Pandy (globulins) negative.
- Has less than 5 cells / mm^3.
- Has a glycorrachia between 50 and 85 mg% (60 to 80 % of the glycemia).
- Never shows germs.

Recapitulation table of the most important characteristics of the different forms of meningitis :

Parameter	Bacterial Meningitis	Viral Meningitis [15]	Meningitis Tuberculosis	Cryptococcal meningitis	Trypanosomiasis (Stage 2)
Pressure	+ + + +	N to +	+ + +	++++	+++
Aspect	Turbid or festering	Clear	Clear or yellowish	Clear or slightly turbid	Clear or Turbid
Pandy (globulins)	+	-	+	Variable	+
WBC count	200 to 20.000/mm^3	10 to 700/mm^3	30 to 400/mm^3	Variable	5 to 1.000/mm^3
Type of cells	Neutrophils [16]	Lymphocytes [17]	Lymphocytes [18]	Variable	Lymphocytes
Germs	Meningococci Pneumococci *H. influenzae* …	Not found	*M. tuberculosis* (exceptionally found) [19]	*Cryptococcus neoformans*	Trypanosomes
Proteinorachia	Highly increased 100 – 1000 mg%	Not or slightly Increased 40 – 100 mg%	Increased >100 mg%	Variable	Increased
Glycorachia [19]	Decreased	Normal	Decreased	Normal	Decreased

Mostly, the investigations are done in the onset of the meningal syndrome. In that case, there is no absolute rule for the number or the type of cells found in relation to the origin of the meningitis.

N.B. : In the cerebral malaria, the CSF examination is often normal, with exceptionally a low lymphocytosis. The proteinorachia is increased and the glycorachia is decreased. One can also find a low turbid CSF with some increase of granulocytes number.

Exceptionally, an amoeba (*Naegleria fowleri*) can cause devastating meningitis. The portal of entry is nasal by bathing in contagious water. The cerebrospinal fluid is festering with presence of polynuclear cells and bacterial examination is negative. A fresh smear shows vegetative forms: about 8 to 30 µm, moving with « explosive » cytoplasmic pseudopodes with numerous vacuoles.

[15] The spirochetes (syphilis, leptospirosis, Borreliosis) and brucellosis can also display meningitis with clear fluid and the same characteristics as a viral meningitis.
[16] In 30 % of cases, there is predominance of lymphocytes. In partially treated meningitis, a predominance of monomorphonuclear (lymphocytes) cells can often be observed.
[17] In the early stage of viral meningitis, a predominance of neutrophils is seen. Predominance of lymphocytes is only found in installed viral meningitis.
[18] In 30 % of cases, there is a predominance of neutrophils. If the sensitivity of the Ziehl stain on a CSF is low, (< 20 %), its specificity is very high.
[19] Glucose will disintegrate soon after sample collection. Therefore, it is important to perform the glucose measurement as soon as possible.

MEASURING OF CSF GLOBULIN CONCENTRATION WITH PANDY REACTION

PRINCIPLE :

Globulin precipitation in presence of phenol. Because of its positivity threshold, the test is only useful on CSF.

MATERIAL :

Material for lumbar puncture + sterile tubes, hemolyse tubes, Pandy reagent, Pasteur pipette, black cardboard.

METHOD :

1. Transfer 1 ml of the Pandy reagent in a small reagent tube.
2. Place the tube in front of a black cardboard.
3. Add, drop by drop, 3 drops of CSF.
4. Control the tube after each drop.

INTERPRETATION OF THE RESULTS:

If white precipitation is formed while the drops are getting in contact with the reagent, the test is positive (presence of an important amount of globulins).
The test is negative if no white precipitation is formed, if the mixture stays clear or if a slight turbidity appears but immediately dissolves.

ANSWER TYPES :

Pandy test negative.

Pandy test positive.

USE OF TRANS-ISOLATE (TI) MEDIUM :

A diphasic medium Trans-Isolate medium that is developed for the transport of primary cultures of cerebrospinal fluid samples from patients with bacterial meningitis. It consists of a charcoal-starch agar slant and soybean-casein digest-gelatin broth buffered at pH 7.2 with 0.1 M 3-(N-morpholino) propane sulfonic acid buffer.
In the laboratory, this medium supports the growth and survival of stock cultures of *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* for at least 3 months.

Free bottles of the medium can be obtained from Statens Institute for Folkehelse [SIF], Geitmyrsveien 75, 0462 Oslo 4, Norway Fax : + 47 22.04.25.18. They are preferably conserved between 2°C et 8°C and must be used within 6 months after the indicated date of fabrication. Before use, control the macroscopic aspect of the medium : the liquid must be clear and no colonies may be visible on the black agar. Discard all media that show contamination signs: cloudy liquid and/or presence of colonies on the agar.

Under field conditions in Africa, cerebrospinal fluid samples from patients suspected of having bacterial meningitis were inoculated directly onto plates of chocolate agar medium and into bottles of Trans-Isolate medium. An aetiological agent was isolated from 52 spinal fluids by direct plating. After shipment to Atlanta, Ga., 2 to 4 weeks later, the same etiological agents were recovered from 38 bottles of Trans-Isolate medium.

MATERIAL :

Material for lumbar puncture + sterile tubes, syringe of 2 ml, 21 G needle, 19 G needle, cotton, tape, transport medium for biological specimen.

METHOD :

1. Collect the CSF in a sterile tube. The inoculation of the medium must be executed within one hour of the sampling. Inoculation is useless if the patient has started an antibiotic treatment within 24 hours preceding the lumbar puncture.
2. Warm up the TI medium (to room temperature).
3. Disinfect the rubber button of the vial with alcohol and let dry.
4. Aspire from the sterile tube 0.1 to 0.5 ml of the CSF with a green 21 G needle.
5. Inject the CSF through the button of the TI vial.
6. After injection, ventilate the vial by bringing in a big needle 19 G into the button.
7. Close the end of the needle with hydrophilic cotton to avoid contaminations. Hold the cotton with a tape.
8. Keep the medium ventilated with the needle until the day of transport (maximum 3 weeks). The ventilated media are stored at room temperature (avoid temperature superior than 40°C) away from direct light until the moment of transport. Never refrigerate. It is important to keep the aerated media at least 2 or 3 days before shipping to allow the bacteria to grow.

Before shipment, withdraw the ventilation needle, pack the vials according to the prescriptions for transport of bio hazardous samples scrupulously and add all necessary administrative and clinical information (name, date, place of collection, country,....) Transport is performed at room temperature and may last 1 week without any problem.

Store of Trans-Isolate (TI) inoculated medium with CSF :

Storage	Before shipment Ventilated medium with a needle	During shipment Non ventilated medium (take out the needle)
Delay	3 weeks	1 week
Temperature	Room temperature **Never refrigerate**	Room temperature **Never refrigerate**

METHOD FOR AGGLUTINATION TESTS WITH LATEX PARTICLES:

PRINCIPLE :

During infection, some bacteria produce natural polysaccharide antigens, which can be recognized with immunological techniques. The reagents are composed of latex particles, which are coated with specific antibodies. This method allows antigen detection in CSF, by means of a rapid agglutination reaction on a cart.

Commercialised agglutination reagents for latex particles for *Neisseria meningitidis*, *Streptococcus pneumoniae,* Streptococci of group B, *Escherichia coli* K1 and *Haemophilus influenzae.* (WHO). The price in 2004 for a kit of 25 identification tests for 5 bacteria Slidex was about 120 €.

Test	Fournisseur*	Information sur le produit
Directigen	Becton-Dickinson Microbiology Systems	Code 4950160 : *N. meningitidis* groupes A/Y et C/W135 Code 4952260 : *H. influenzae* type b Code 4951960 : *S. pneumoniae* Code 4952360 : Meningitis Combo Test Kit pour *N. meningitidis* groupes C/W135, A/Y, B/E. *coli* K1, *H. influenzae* type b, *S. pneumoniae*, et streptococcus groupe B
MicroScan	VWR Scientific	Code B1049-6 ImmunoSCAN Direct : *S. pneumoniae* Latex Test Kit Code B1049-7 ImmunoSCAN Direct : Meningitis Test Kit pour *N. meningitidis* groupes A/C, Y/W135
Pastorex	Sanofi Diagnostics Pasteur	Code 61701 : Pastorex Meningitis Kit pour *N. meningitidis* groupes A, B, C, Y, W135, *H. influenzae* type b, *S. pneumoniae* et streptococcus groupe B
Slidex	BioMérieux	Code 58 803 : Slidex meningite Kit 5 pour la recherche de *N. meningitidis* groupes A, C, B/E. coli K1, *H. influenzae* type b et *S. pneumoniae*
Wellcogen	Murex Diagnostics, Inc.	Code ZL26 : Wellcogen Bacterial Antigen Kit pour la recherche de *N. meningitidis* groupes A/C/Y/W135, B/E. *coli* K1, *H. influenzae* type b, *S. pneumoniae* Code ZL21 : Wellcogen *H. influenzae* type b et streptococcus groupe B Code ZL22 : Wellcogen *S. pneumoniae* Code ZL23 : Wellcogen *N. meningitidis* groupes A/C/Y/W135 Code ZL24 : Wellcogen *N. meningitidis* B/E. coli K1

Always follow punctually the instructions of the manufacturer of the used kit. The expiry date of the kits is from 3 to 9 months after production, according to the types of reagents. (3 months for the pneumococci). Some general recommendations must be taken into account : The CSF must be examined as soon as possible, in order to obtain reliable results. If the examination must be delayed for some hours, the specimen may be stored at 2°C to 8°C. For a longer delay, the sample must be frozen. Bacterial culture is excluded in that case. The reagents must be stored in the refrigerator (between 2°C and 8 °C) when they are not used. The deterioration of reagents will accelerate in higher temperature conditions, especially in tropical climates. In this case, the tests may become non reliable before the expiry date of the kit. The latex suspensions may never be frozen.

The warming up of the CSF at 100 °C allows to increase the sensitivity of the test by better releasing antigens. **Execute, before warming of the CSF, all the other necessary bacteriological tests.** Centrifugation of the CSF increases the specificity by separating the soluble antigens from other particles, which can interfere with the reaction.

The global specificity is rather good (in the range 98 %). Sensitivity is in the range of 75 % for *Neisseria meningitidis*, 90 % for *Streptococcus pneumoniae,* and 85 % for *Haemophilus influenzae.* The sensitivity profit compared to gram is rather low.

Based on the same principle, a latex agglutination serology test for antigen detection of *Cryptococcus*, as well in serum as in CSF, can be purchased. (example Crypto-LA de Fumouze, 17 € for 70 tests in 2004).

MATERIAL :

Material for lumbar puncture + latex kit, centrifuge, sterile centrifugation tubes with conical bottom, boiling water bath, [rotating mixing apparatus], Pasteur pipettes, 30 µl drop dispenser [or automatic pipette of 30 µl with tips], timer.

METHOD :

1. Warm up the CSF in a boiling water bath during 5 minutes.
2. Centrifuge the CSF during 10 minutes at 2.000 revolutions per minute (RPM).
3. Collect the supernatant.
4. Bring the reagents at room temperature.
5. Homogenise slightly the latex suspension(s) and empty the drops which stayed in the drop dispenser.
6. Put a drop of each latex suspension on a disposable card.
7. Bring 30 µl of CSF near each drop of latex suspension.
8. Mix the reagent with the CSF with a plastic agitator. Use the complete reaction zone.
9. Balance the card during 2 minutes [or use a rotator].
10. Read the reaction under a strong light, without magnification.

INTERPRETATION OF RESULTS

Negative reaction :

- Absence of agglutination after 2 minutes. The suspension stays homogenous and slightly milky.

Positive Reaction:

- Appearance of agglutination with only one reagent within 2 minutes. This proves the presence in the CSF of the correspondent antigen.

Non interpretable results :

- Reaction after more than two minutes.
- Absence of agglutination of the latex reagent for the positive control.
- Presence of agglutination with a latex reagent for the negative control.
- Presence of agglutination with 2 (or more) latex reagents (mixed infection, but extremely rare?).

URETHRITIS

PRINCIPLE :

The diagnosis of urethritis in man is more and more based on clinical algorithms. A microscopic examination after methylene blue stain on an urethral specimen can be used. This technique permits differentiation of a gonococcal urethritis (G.U., caused by *Neisseria gonorrhoeae*), from non gonococcal urethritis (N.G.U., caused by *Chlamydia trachomatis*, ...). The additional (or principal ?) benefit of this examination is to maintain a laboratory competence in the examination of *Neisseria*, necessary in the context of meningitis.

SAMPLING :

The examination is done on an urethral pus specimen. Prosecute, if possible, the sample collection in the early morning, before the patient has urinated. If the urethra is unclean, it should be cleaned with a compress that is drenched in physiological water.

METHOD :

1. Notify the patient number on a new slide.
2. Flame a new slide by passing it 3 x through an alcohol lamp flame.
3. Verify if the wire loop is quite flat, to avoid to injure the patient during the collection.
4. Drench the wire loop in the bottle with the sand/cresyl mixture.
5. Flame the wire loop (bring the wire to red-hot over his whole length) next let cool down.
6. Press slightly the penis to make appear a drop of pus at the urinary channel.
7. Take off the pus with the sterile platinum wire loop. When no pus appears, penetrate the wire loop for about 2,5 cm in the urethral canal in order to obtain some sample.
8. Spread the pus over the flamed slide, making so an as thin as possible smear covering the greatest part of the slide.
9. Drench the wire loop in the bottle with the sand/cresyl mixture.
10. Flame the wire loop (bring the wire to red-hot over his whole length) next let cool down.
11. Notify the aspect and predominance of the urethral secretion in the examination logbook.
12. Fixate the smear with methanol and stain it with methylene blue.
13. Observe the smear under the microscope with immersion oil (objective 100x, eyepiece 10x) for examination of neutrophils. Estimate the number of neutrophils per field in 5 fields [Count the number of neutrophils in 5 fields, then divide the result by 5]. Search subsequently intra- or extra cellular coffee bean shaped diplococci.

ANSWER TYPES :

- Aspect and amount of urethral discharge :
- Quantity of neutrophils: _ _ _ leucocytes per field.
- **or** negative examination.
  or presence of intra cellular diplococci (or intra and extra cellular).
  or presence of extra cellular diplococci .

INTERPRETATION OF RESULTS:

A gonococcal urethritis (G.U.) is mostly characterised by a festering discharge, while a mucous discharge is more associated with a non gonococcal urethritis (N.G.U.).
An infection is called acute if meanly more than 4 neutrophils per microscopic field are found.
In male persons, the presence of intra cellular diplococci supplies a reliable diagnosis, while presence of extra cellular diplococci provides an almost certain diagnosis.

On a vaginal smear, or for an urinary specimen, the sensitivity and specificity of this technique are about 50 %

PREPARATION OF REAGENTS

1. ACID ALCOHOL for Ziehl 3 % v/v
(unstaining solution for mycobacteria, technique of warm Ziehl-Neelsen).

This is the first choice reagent. It can be replaced by Sulfuric Acid 20 %.

☠ **ATTENTION:** Ethanol is inflammable; manipulate this product away from fire. Hydrochloric acid is extremely corrosive. Vapours of hydrochloric acid are toxic. This product must be manipulated with extreme care and with open windows.

Ethanol 96 %.....	970 ml
Hydrochloric Acid.....	30 ml

Pour 970 ml of Ethanol 96 % in a flask of one litre. Add slowly 30 ml of hydrochloric acid while letting it flow along the wall. Mix the continence witch will warm up. These reagents may be of technical grade.

CONSERVATION: This can be stored at room temperature for six to twelve months.
STORAGE: Brown or white flask of 1000 ml.
Identification: **ACID ALCOHOL for ZIEHL** and notify the date of preparation.

2. ALCOHOL ACETONE 90 % (v /v)
(solution for Gram decolorization)

This reagent can be replaced by alcohol 96 %. It may be remarked that the composition of the Gram decolorization product varies from one author to the other, from pure acetone to ethanol. The stronger the acetone concentration the faster and more drastic the decolorization will elapse. The basic products may be of technical quality.

☠ **ATTENTION :** Ethanol is inflammable; manipulate this product away from fire. Acetone is harmful and extremely inflammable. Acetone vapours are irritating. Manipulate this product with open windows.

Ethanol 96 %.....	900 ml
Acetone.....	100 ml

CONSERVATION : Some years in an hermetically closed flask.
STORAGE: Brown or white flask of 250 ml.
Identification: **ALCOOL ACETONE** and notify the date of preparation.

3. CRISTAL VIOLET OR GENTIAN VIOLET *(for Gram stain)* :

☠ **ATTENTION :** Ethanol is inflammable, manipulate this product away from fire.

♦ **Stock solution A :**

Cristal Violet or Gentian Violet .....….	25 g
Ethanol 95 %.....	250 ml

The continence of a flask of 25 g Gentian Violet is introduced into a brown bottle of 250 ml, next filled with Ethanol 96 %. The bottle is thoroughly shaken three times in the same day. Let repose. The solution is ready to use the next day.
N.B. Cristal violet can be dissolved in methanol instead of in Ethanol. There is no need to wash or rinse the flasks with saturated solutions. Just add from time to time some dye and some alcohol. As long as there is some powder on the bottom of the flask, the supernatant is saturated.

CONSERVATION : Some years in a brown, hermetically closed bottle
STORAGE : Brown flask of 500 ml
Identification : **CRISTAL VIOLET SOLUTION A** and notify the date of preparation.

♦ **Stock solution B** :

Ammonium Oxalate $(NH_4)_2C_2O_4.H_2O$..... 5 g
Distilled water..... 500 ml

CONSERVATION : 2 to 3 months in an hermetically closed bottle.
STORAGE: brown or white 500 ml
Identification: **CRISTAL VIOLET SOLUTION B** and notify the date of preparation.

CRISTAL VIOLET OR GENTIAN VIOLET (working solution for Gram stain) :

Mix 100 ml of solution A with 400 ml of solution B. Store in a brown flask. Let stand for 24 hours. Filter on a filter paper before bringing it into a brown flask, protected against light.

CONSERVATION : Stable for several months in a brown, hermetically closed bottle.
STORAGE : A brown flask of 500 ml.
Identification: **CRISTAL VIOLET** and notify the date of preparation
Filter before use.

N.B. This reagent is also commercialised in a ready to use form [example Bio Mérieux 55545]

4. FUCHSIN FOR ZIEHL
(For staining of mycobacteria, hot Ziehl-Neelsen technique)

☠ **ATTENTION :** Phenol is extremely corrosive and toxic. This product must be manipulated with extreme care.

♦ **Fuchsin, saturated stock solution:**

Basic Fuchsin ..... 25 g
Ethanol 96 %..... 250 ml

The continence of a 25 g flask of basic Fuchsin (example Fluka 602) is brought in a brown bottle of 250 ml, next filled with ethanol 96 %. Shake the bottle thoroughly three times during the same day and leave it until next day. Then the solution is ready to use. Better results are obtained with neo fuchsin (example new fuchsin Merck 1.04041.0025), but the price is much higher.

P.S. Basic Fuchsin can be dissolved in methanol instead of in ethanol. It is not necessary to rinse or wash the flasks with saturated solutions. Just add from time to time some dye and some alcohol. As long as there is some powder on the bottom of the flask the solution can be considered as saturated.

CONSERVATION: This can be stored at room temperature for six to twelve months.
STORAGE: Brown flask of 250 ml
Identification: **SATURATED BASIC FUCHSIN SOLUTION** and the date of preparation.

♦ **Aqueous stock Phenol solution 5 % (v/v) :**

Phenol crystals……..... 50 ml
Distilled water..... 950 ml

The water must be distilled or filtered. Tab water often contains saprophytic mycobacteria which can't be microscopically distinguished from pathogen mycobacteria!
Normal Phenol crystals are colourless. When their colour is rose violet, they are expired and may no longer be used. Aqueous phenol solution 5 % is prepared by adding 50 ml Phenol melted crystals at 45 °C (bath or sun, or flask of 50 g de phenol [example : Fluka 77610]) with 950 ml distilled water. The cylinder for measuring the volume of phenol must be warmed up in a bath or in the sun (if this isn't done, the phenol will immediately crystallize in the cylinder).

CONSERVATION: Several months in a hermetically closed flask.
STORAGE: Brown flask of 1000 ml
Identification: **AQUEOUS PHENOL SOLUTION** 5 % and notify the date of preparation.

ZIEHL FUCHSIN (work solution) :

Saturated solution of filtered Basic Fuchsin	100 ml
Phenol, aqueous solution 5 %.....	900 ml

CONSERVATION : At least 2 years.
STORAGE : A glass brown flask of 1000 ml.
Identification : **ZIEHL FUCHSIN** and notify the date of preparation.
Filter before use.

!! Implement a quality control with positive and negative samples for AFB !!

5. DILUTED FUCHSIN *(for Gram stain)* :

Diluted Fuchsin for Gram can be replaced with Safranine for Gram (reagent 10). Safranin is superior to fuchsin, since it realises a better contrast staining with Cristal violet.

☠ **ATTENTION :** Phenol is extremely corrosive and toxic. Manipulate this product with great precaution.

Phenol Fuchsin for ZIEHL (reagent 6).....	1 ml
Distilled water.....	9 ml

STORAGE : 1 month in the best conditions.
Make the dilution before every coloration.

6. LUGOL DILUTED (for Gram stain) :

☠ **ATTENTION :** Sublimated Iodine is harmful by inhalation or contact.

Potassium Iodide (KI).....	2,34 g
Iodine, crystal or sublimated	1,66 g
Distilled water.....	500 ml

Dissolve Potassium Iodide in 10 ml distilled water. Add Iodine crystals (previously pulverised in a mortar). Mix until it is dissolved. Add the rest of distilled water (490 ml). N.B. : iodide does not dissolve in water and badly in potassium iodide. The dissolution of iodide is even much more difficult if it contains impurities. Therefore always use sublimated bi iodide bi or highly purified (suprapur).
Store in a brown flask, protected against light.

CONSERVATION : not filtered : 3 months. Lugol must have a red-brown colour. If it is yellow, it is no longer active and must be discarded.
STORAGE : A brown, glass flask of 500 ml.
Identification : WEAK LUGOL FOR GRAM and notify the date of preparation.
Filter before use.

N.B. This reagent is also commercialised in a ready to use form [example Bio Merieux 55546]

7. METHYLENE BLUE

(for mycobacteria and bacteriology)

♦ **Methylene Blue, stock solution:**

Methylene Blue	25 g
Ethanol 96 %.....	250 ml

The continence of a flask of 25 g of methylene blue is brought in a brown bottle of 250 ml and then filled with ethanol 96 %. Shake the bottle thoroughly three times during the same day and leave it until next day. Then the solution is ready to use. One can add ethanol as long as there is methylene blue deposit in the bottle.

N.B. : Methylene blue can also be dissolved in me*thanol instead of in ethanol. It is not necessary to rinse or wash the flasks with saturated solutions. Just add from time to time some stain and some alcohol. As long as there is some powder on the bottom of the flask the solution can be considered as saturated.*

CONSERVATION: This can be stored at room temperature for six to twelve months.
STORAGE: Brown or white flask of 250 ml

Identification: SATURATED METHYLENE BLUE and notify the date of preparation.

♦ **Methylene Blue, working solution for mycobacteria and bacteriology:**

Filtered, saturated methylene blue solution.....	100 ml
Distilled water.....	900 ml

CONSERVATION: At least 1 year in a hermetically closed brown flask.
STORAGE: Brown flask of 1000 ml.

Identification: METHYLENE BLUE and notify the date of preparation. **Filter before use.**

8. PANDY REAGENT (for detection of globulins in spinal fluid.) :

☠ **ATTENTION :** Phenol is extremely corrosive and toxic. Manipulate this product with great care.

Phenol	50 g
Distilled water.....	500 ml

Crystallised Phenol is normally colourless. When the colour is pink violet, it is expired and may no longer be used. There are packages of 50 g [example : Fluka 77610]

The Pandy reagent is a saturated phenol solution. Bring the Phenol in a brown flask of 1000 ml. Add distilled water. Agitate intensely. Let repose for 1 day. Verify that enough non dissolved phenol remains. If so, filter and store in a brown flask. If all the phenol is dissolved, add 10 g Phenol and wait one more day before filtering.

CONSERVATION : some years in an hermetically closed brown bottle.
STORAGE : a brown, glass bottle of 1000 ml.

Identification : PANDY REAGENT and notify the day of preparation

9. SAFRANINE (for Gram stain) :

Safranine for Gram may be replaced by diluted Fuchsin for Gram (reagent 15). Safranine is superior to fuchsin, since it realises a better contrast staining with Cristal violet.

☠ **ATTENTION :** ethanol is inflammable. Manipulate this product away from fire

♦ Safranin, saturated stock solution :

Safranine O.....	25 g
Ethanol 96 %	500 ml

The continence of a 25 g Safranine flask is brought into a brown bottle of 500 ml, next filled with ethanol 96 %. Shake the bottle thoroughly three times during the same day and leave it until next day. Then the solution is ready to use. Ethanol must be added if no deposit of Safranine rest on the bottom.

P.S. Safranine may be dissolved in Methanol instead of in ethanol. It is not necessary to rinse or wash the flask with saturated solutions. Just add from time to time some dye and some alcohol. As long as there is some powder on the bottom of the flask the solution can be considered as saturated.

CONSERVATION: Some years in a brown, hermetically closed bottle.
STORAGE: a brown, glass flask of 500 ml.
Identification: SATURATED SAFRANIN and notify the date of preparation.

SAFRANIN FOR GRAM (work solution) :

Saturated safranin (supernatant).....	200 ml
Distilled water.....	800 ml

CONSERVATION : Some months in a brown, hermetically closed bottle.
STORAGE : a brown, glass flask of 500 ml.
Identification : SAFRANIN FOR GRAM and notify the date of preparation.

N.B. This reagent is also commercialised in ready to use form [example Bio Mérieux 55548]

10. SULFURIC ACID 20 % (v/v)
(unstaining solution for mycobacteria - Hot Ziehl Technique).

This is the second choice reagent. Only used when depletion of stock of hydrochloric acid.

☠ **ATTENTION:** Sulfuric acid is extremely corrosive. Vapours of sulfuric acid are toxic.

Distilled water.....	800 ml
Sulfuric Acid.....	200 ml

Never pour water in an acid solution. Addition of a small quantity of water in an acid produces enough heath to make the bottle explode!
Pour 800 ml distilled water in a one-litre flask. Measure 200 ml of sulfuric acid in a cylinder of 250 ml. Add slowly 200 ml of sulfuric acid with quantities of 50 ml a time, letting flow the acid on the wall of the flask. Mix the continence witch will warm up. These reagents may be of technical quality.

CONSERVATION: This can be stored at room temperature for six to twelve months.
STORAGE: Brown or white flask of 1000 ml
Identification: SULFURIC ACID 20 % and notify the date of preparation.

PREPARATION OF REAGENTS FOR AURAMINE STAINING

1. ACID ALCOHOL for Auramine 0.5% v/v
(unstaining solution for mycobacteria, technique of Auramine).

☠ **ATTENTION:** Ethanol is inflammable; manipulate this product away from fire. Hydrochloric acid is extremely corrosive. Vapours of hydrochloric acid are toxic. This product must be manipulated with extreme care and with open windows.

Ethanol 96 %.....	995 ml
Hydrochloric Acid.....	5 ml

Pour 995 ml of Ethanol 96 % in a flask of one litre. Add slowly 5 ml of hydrochloric acid while letting it flow along the wall. Mix the continence witch will warm up. These reagents may be of technical grade. Ethanol can be replaced by Methanol.

CONSERVATION: This can be stored at room temperature for six months.
STORAGE: Brown or white flask of 1000 ml.
Identification: ACID ALCOHOL 0.5% and notify the date of preparation.

2. AURAMINE SOLUTION
(For staining of mycobacteria, Auramine technique)

☠ **ATTENTION:** Ethanol is inflammable; manipulate this product away from fire. Phenol is extremely corrosive and toxic. This product must be manipulated with extreme care.

- **Auramine stock solution 1 %:**

Auramine O, dye component >85% ...	1 g
Ethanol 96 %.....	100 ml

1 g of Auramine (example Merck 1.012010) 602) is brought in a brown bottle of 100 ml, next filled with 100 ml ethanol 96 %. Shake the bottle thoroughly three times during the same day and leave it until next day. Then the solution is ready to use. *P.S. Auramine can be dissolved in methanol instead of in ethanol.* The auramine must be of analytical grade with a purity of> 85% .DO NOT HEAT: Auramine is inactivated by a temperature> 40 ° C.

CONSERVATION: This can be stored at room temperature for 3 months.
STORAGE: Brown flask of 100 ml
Identification: **AURAMINE 1%,** stock solution and notify the date of preparation.

- **Aqueous stock Phenol solution 3.3 % (v/v):**

Phenol crystals……....	30 ml
Distilled water.....	870 ml

The water must be distilled or filtered. Tab water often contains saprophytic mycobacteria which can't be microscopically distinguished from pathogen mycobacteria! Normal Phenol crystals are colourless. When their colour is rose violet, they are expired and may no longer be used. Aqueous phenol solution 3.3 % is prepared by adding 30 ml Phenol melted crystals at 45 °C (bath or sun, or flask of 50 g de phenol [example: Fluka 77610]) with 870 ml distilled water. The cylinder for measuring the volume of phenol must be warmed up in a bath or in the sun (if this isn't done, the phenol will immediately crystallize in the cylinder).

CONSERVATION: Several months in a hermetically closed flask.
STORAGE: Brown flask of 1000 ml
Identification: **AQUEOUS PHENOL SOLUTION 3.3 %** and notify the date of preparation.

- **AURAMINE 0.1 % (work solution)**

Auramine 1%, stock solution…….....	50 ml
Phenol, aqueous solution 3.3 %.....	450 ml

CONSERVATION: 1 month.
STORAGE: A glass brown flask of 500 ml.
Identification: Auramine 0.1%,working solution and notify the date of preparation.
Filter before use.

Implement a quality control with positive and negative samples for AFB.

3. KOH SOLUTION 20 %

☠ **ATTENTION:** Potassium hydroxyde is extremely corrosive. This product must be manipulated with extreme care

Potassium hydroxide (KOH).....	20 g
Distilled water.....	100 ml

Dissolve the potassiumhydroxyde in the distilled water. During this process the solution will heat up (exothermal reaction)!

CONSERVATION: This can be stored at room temperature for 24 months.
STORAGE: Brown or white flask of 100 ml
Identification: **POTASSIUM HYDROXYDE 20%** and notify the date of preparation.

4. METHYLENE BLUE (Löfflers)
(counterstaining solution for mycobacteria, technique of Auramine).)

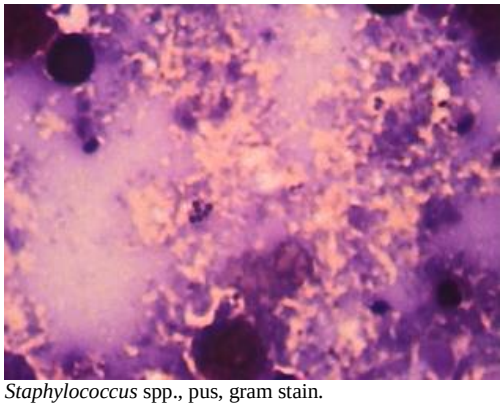
☠ **ATTENTION:** Ethanol is inflammable; manipulate this product away from fire. Potassiumhydroxyde is extremely corrosive. This product must be manipulated with extreme care

Methylene Blue	5 g
Ethanol 96 %.....	300 ml
Potassium hydroxide 20%	1 ml
Distilled water.....	695 ml

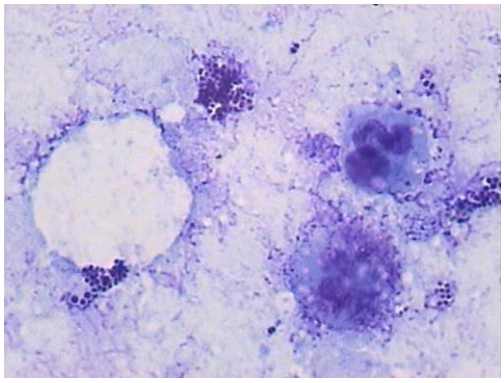
5 g of Methylene blue is brought in a brown bottle of 1000 ml and then filled with 300 ethanol 96 %. Shake the bottle thoroughly three times during the same day Add the potassium hydroxide and distilled water and mix well. Then the solution is ready to use. *N.B.: Methylene blue can also be dissolved* in m*ethanol instead of in ethanol.*

CONSERVATION: This can be stored at room temperature for twelve months.
STORAGE: Brown or white flask of 1000 ml
Identification: **LOFFLER METHYLENE BLUE** and notify the date of preparation.

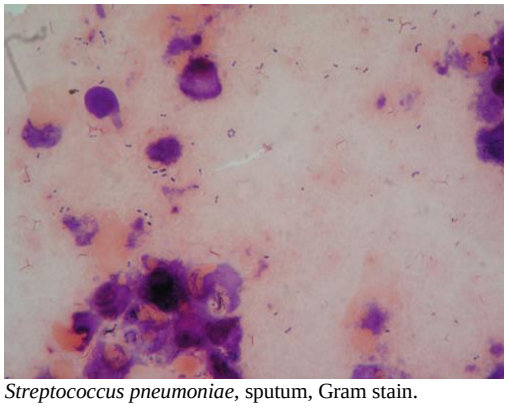
Important human pathogen bacteria



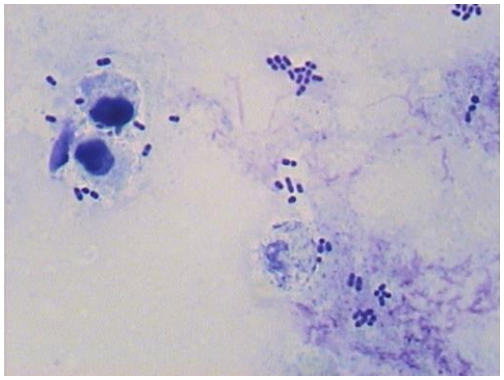
Staphylococcus spp., pus, gram stain.



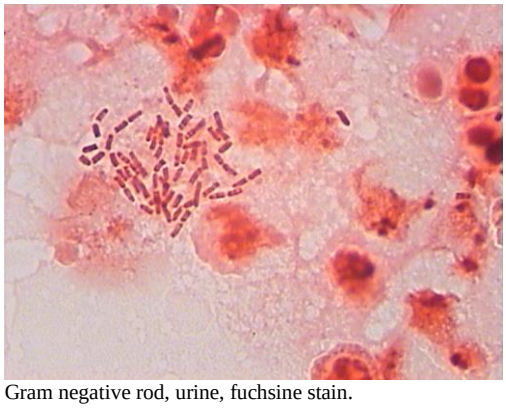
Staphylococcus spp., pus, methylene blue stain.



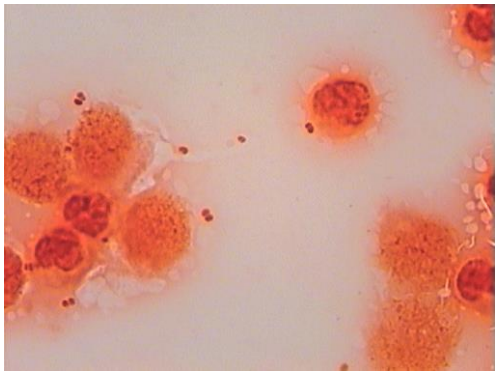
Streptococcus pneumoniae, sputum, Gram stain.



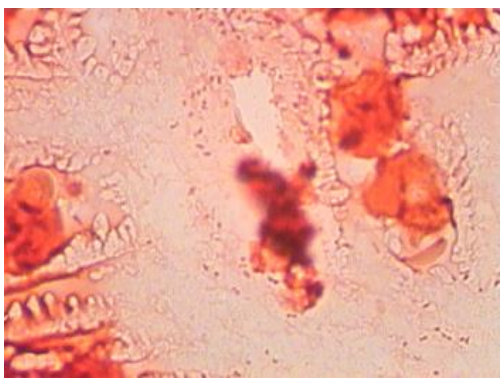
Streptococcus mutans, pus, methylene blue stain.



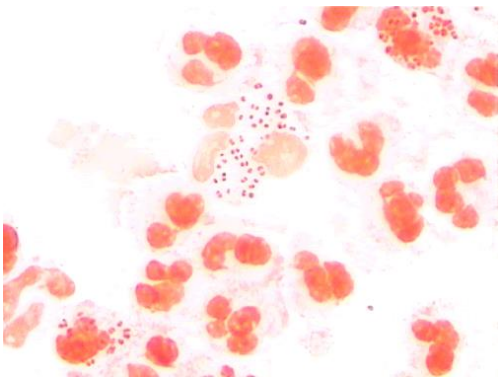
Gram negative rod, urine, fuchsine stain.



Neisseria meningitidis, CSF, fuchsine stain.

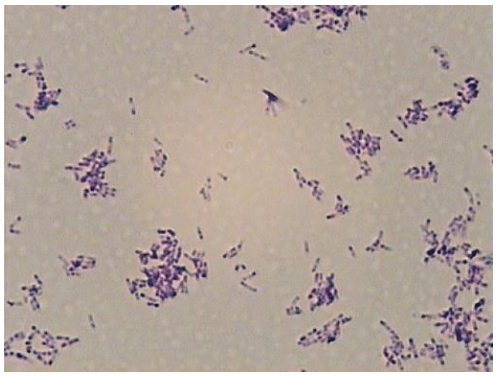


Haemophilus influenzae, CSF, fuchsine stain.

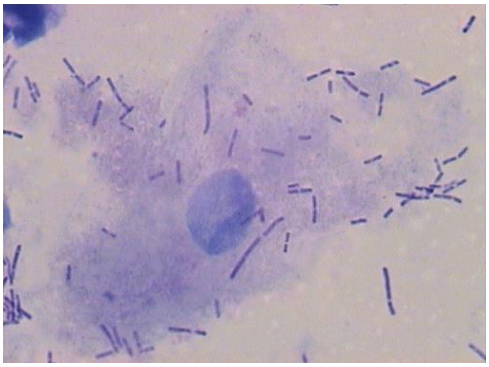


Neisseria gonnorrhoeae, urethral discharge, safranine stain.

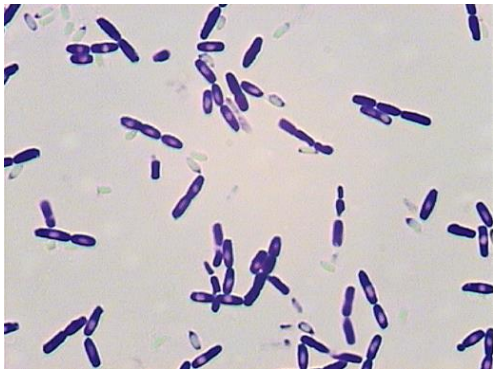
All stained slides are viewed with ocular 10x, and with oil immersion objectif 100x



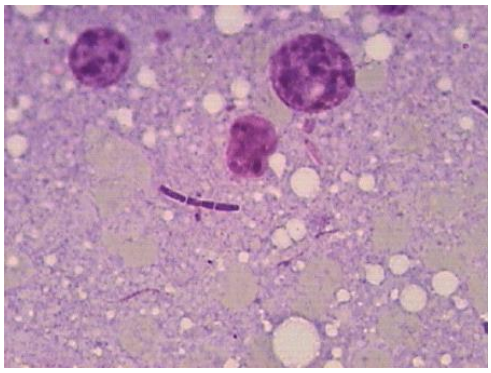
Corynebacterium diphteriae, culture, Albert stain.



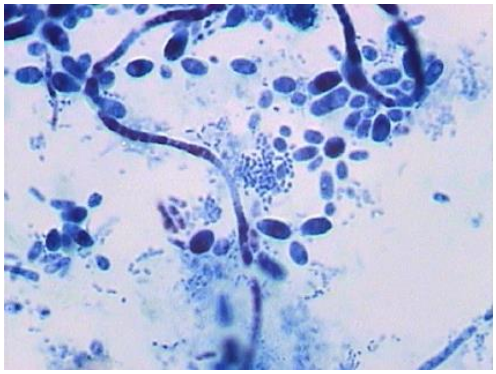
Lactobacillus acidophilus, vaginal discharge, methylene blue stain.



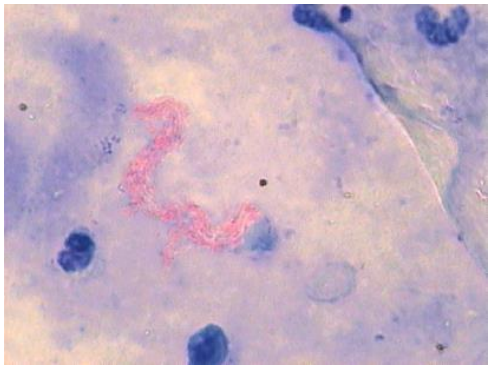
Bacillus subtillis, sporulating bacteria, culture, crystal violet stain.



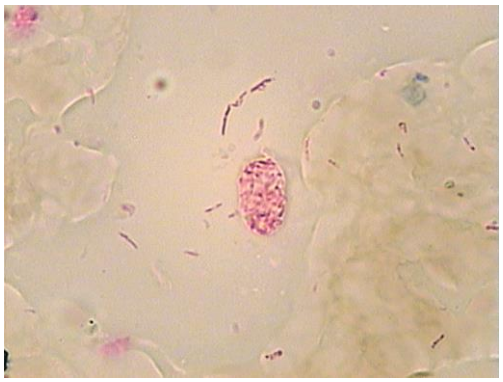
Bacillus anthracis, Deps, mice liver, Giemsa stain



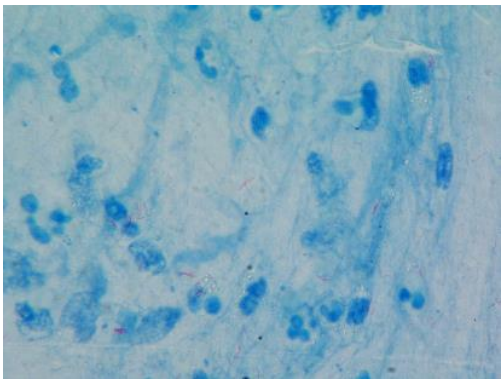
Candida spp.(yeast) and vaginal bacteriae, vaginal discharge, methylene blue stain.



Mycobacterium tuberculosis, cords, sputum, Ziehl stain.



Mycobacterium leprae, globus, skin, Ziehl stain



Mycobacterium tuberculosis, sputum, Ziehl stain.

All stained slides are viewed with ocular 10x, and with oil immersion objectif 10

Some useful references and internet-sites

TUBERCULOSIS

- For WHO policy guidance on TB diagnostics and laboratory strengthening:

  http://www.who.int/tb/laboratory/en/

- For WHO and GLI TB training packages, guidelines, resources:

  http://www.stoptb.org/wg/gli/documents.asp

- For OSHA (American protection agency) and CDC guidance, training videos, standards of respiratory protection:
  https://www.osha.gov/SLTC/respiratoryprotection/index.html
  http://www.cdc.gov/niosh/topics/respirators/

- For 3M video instructions on how to perform a fit test:
  http://solutions.3m.co.uk/wps/portal/3M/en_GB/PPE_SafetySolutions_EU/Safety/Products/RespiratoryProtection/DisposableRespirators/

Laboratory services in tuberculosis control. Part I: Organization and management. Geneva, World Health Organization, 1998.

Laboratory services in tuberculosis control. Part II: Microscopy. Geneva, World Health Organization, 1998.

Laboratory services in tuberculosis control. Part III: Culture. Geneva, World Health Organization, 1998.

Tuberculosis Laboratory biosafety manual. Geneva, World Health Organization, 2012.

Laboratory Diagnosis of Tuberculosis by sputum microscopy. The handbook. SA Pathology, 2013.

Mycobacteriology Laboratory Manual, Global Laboratory Initiative GLI, 2014.

Ventilated Workstation Manual for AFB smear microscopy, GLI, 2011

MENINGITIS

Laboratory Methods for the Diagnosis of Meningitis Caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae.* Geneva, World Health Organization, 2011.

Instituut voor Tropische Geneeskunde
Institut de Médecine Tropicale
Institute of Tropical Medicine
Instituto de Medicina Tropical

Nationalestraat, 155
B – 2000 Antwerpen

Stichting van Openbaar Nut 0410.057.701

POSTGRADUATE IN TROPICAL MEDICINE AND INTERNATIONAL HEALTH
MODULE 1 & 2
CLINICAL & BIOMEDICAL SCIENCES OF TROPICAL DISEASES

TROPICAL HAEMATOLOGY

(Practical notes)

SEPTEMBER 2018

Philippe Gillet – Idzi Potters – Hilde De Boeck - Jan Jacobs

Table of Contents

GENERAL HAEMATOLOGY

BLOOD TRANSFUSION

BLOOD COLLECTION

CAPILLARY BLOOD

Capillary blood is the cheapest and the easiest method for blood collection. Capillary blood is mainly used when the volume of required blood is small (up to 100 µl). Disadvantages in using capillary blood for blood tests include:

- Great possibility of sampling errors, particularly when the blood is not free-flowing (dilution of the sample with tissue fluid).
- Difficulty in obtaining sufficient blood (for more than one test).
- Rapid clotting of blood.
- Test cannot be repeated or further tests cannot be performed when results are unexpected.

Capillary blood is obtained by puncturing the skin with a lancet. In adults or children, the best place will be on the 3rd or 4th finger of the left hand, at the side of the finger, which is less sensitive than the tip. In infants, the best place will be the side of the heel or the big toe (The puncture should not be too deep because of the risk of osteomyelitis!). Never collect blood from an infected finger of foot. Never collect blood from an arm in which an intravenous infusion is being given (haemodilution). The puncture should be deep enough to result in free bleeding. A free flow of blood is essential and only the gentlest squeezing is permissible (risk of dilution with tissue fluid resulting in unreliable values).

1. Prepare all supplies in advance: sterile lancet, 2 pieces of cotton wool, one dry, the other soaked with alcohol 70 % and blood collection material (Sahli pipette, slide, capillary tube, …).
2. If possible, ask the patient to clean his hands with soap and hot water (vasodilatation), next dry his hands thoroughly.
3. Slightly massage the place where blood will be taken. Make sure the puncture area is warm enough to allow the blood to flow freely. If necessary, soak the hand or foot of an infant in warm water prior to collecting a sample.
4. Cleanse the puncture area with a cotton swab dipped in 70 % alcohol, let alcohol react for at least 30 seconds, then with a dry cotton to remove any remaining ethanol.
5. Using a sterile lancet, make a rapid puncture, sufficiently deep to allow the free flow of blood. Discard immediately the lancet in a safety container.
6. Execute a slight pressure on the finger to realise a better blood flow.
7. Wipe away the 1st drop of blood with a dry piece of cotton wool since it may contain tissue fluid or disinfectant. (Except for microfilaria detection, since the first drop contains more microfilaria).
8. Press the finger (not too hard) to produce in one time the required amount of blood.
9. When sufficient blood has been collected, press a piece of cotton dipped in 70 % alcohol over the puncture area until bleeding stops.

For glycaemia determination: The use of ether or any disinfectant should be avoided as it may interfere with the reagent strip (glucose oxidase peroxidase reaction). To decrease risk of infection and food contamination (fruit juice, ...) it is therefore important to clean the hands with soap, before blood collection.

VENOUS BLOOD

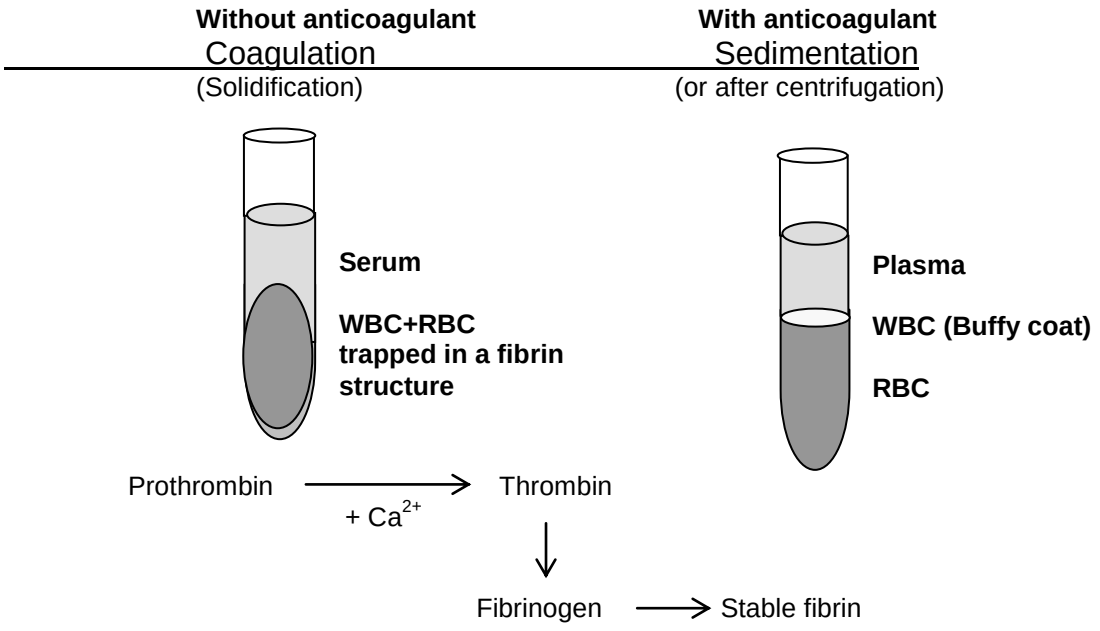
Venous blood is used when more than 100 µl of whole blood is required or when serum from a clotted blood sample is needed. If whole blood is needed, an anticoagulant should be used to prevent clotting and/or morphological blood cell changes. An anticoagulant acts by removing calcium (example: EDTA, trisodium citrate) or by interference with coagulation factors (example : Heparin)

- For most haematological tests (Haemoglobin (Hb), packed cell volume (PCV), white blood cells (WBC) count, blood group determination, …), dipotassium EDTA is recommended.

  Preparation of dipotassium EDTA tubes : Bring 40 µl of a dipotassium EDTA solution (10 g / 100 ml distilled water) in 3 ml tube. Leave the open tubes to dry at room temperature (protect from dust). Close when dry. The correct amount of blood must be added to avoid blood cell changes (2,5 ml). Excess EDTA causes shrinkage and degenerative changes, lack of EDTA will not prevent the coagulation.

- For measuring the erythrocyte sedimentation rate (ESR), trisodium citrate is used to anticoagulate the blood. 4 volumes of venous blood with 1 volume of trisodium citrate 32 g/l.

DIFFERENCE BETWEEN SEDIMENTATION AND COAGULATION



SERUM = PLASMA WITHOUT COAGULATION PROTEINS

HAEMOGLOBIN DETERMINATION

INTRODUCTION

Anaemia is defined as having an amount of haemoglobin below reference values. Different cut-off exist for different age category, gender and pregnant status.

Haemoglobin level to diagnose anemia at sea level (g/l) according to WHO.

Population & age	Non-anemia	Mild anemia	Moderate anemia	Severe anemia
Children 6-59 months	110 or higher	100-109	70-99	Lower than 70
Children 5-11 years	115 or higher	110-114	80-109	Lower than 80
Children 12-14y	120 or higher	110-119	80-109	Lower than 80
Non pregnant (15 years and above)	120 or higher	110-119	80-109	Lower than 80
Pregnant women	110 or higher	100-109	70-99	Lower than 70
Men (15 years and above)	130 or higher	110-129	80-109	Lower than 80

Source: Haemoglobin concentration for the assessment of anemia and assessment of severity, WHO 2011 (http://www.who.int/vmnis/indicators/haemoglobin/en/ viewed 20/12/2016)

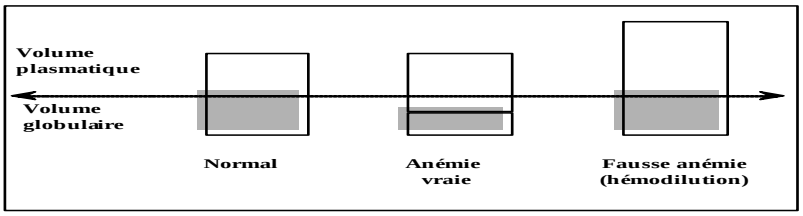
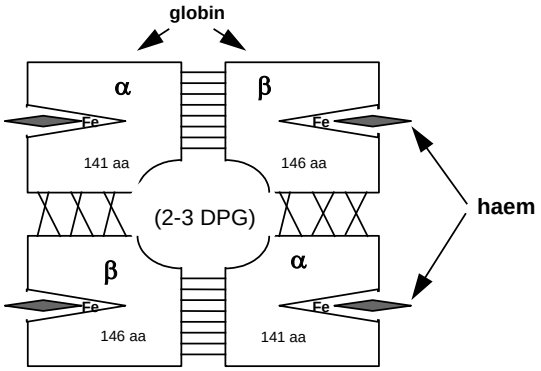


Fig.1 : True and "false" anaemia due to haemodilution. In some circumstances, the reduction of hemogram values is related to a haemodilution caused by plasma excess resulting in a "false anemia": pregnancy, splenomegaly, heart insufficiency, monoclonal immunoglobulines, especially IgM….

Haemoglobin is the most important part of the red blood cell. It is the red pigment that gives the colour to the red blood cells. It carries oxygen and carbon dioxide. Each molecule of haemoglobin contains four linked polypeptide (globin) chains and four haem groups. (Fig. 2). Haem is an iron-containing porphyrin pigment, which is the oxygen carrying part of the haemoglobin molecule. Oxygen binds reversibly with ferrous ions (Fe^{2+}), contained in each haem group. More than 97 % of normal adult haemoglobin is Hb A_1, having 2 *alpha* chains and 2 *beta* chains ($\alpha_2\beta_2$). Up to 3,5 % is HbA_2, consisting of 2 *alpha* chains and 2 *delta* chains ($\alpha_2\delta_2$). Less than 1 % is HbF (foetal) composed of 2 *alpha* chains and 2 *gamma* chains ($\alpha_2\gamma_2$). HbF is the predominant haemoglobin in foetus; untill 3-6 months of life.

Fig 2 : Haemoglobin structure
(Haemoglobin A)



2-3 DPG = 2-3 diphosphoglycerate

The measurement of haemoglobin is important for the diagnosis of the severity of anaemia.
Different techniques, more or less reliable, are available for haemoglobin determination. They are based on different principles, which can be classified in 3 families:

1. Techniques based on the red colour of blood without dilution nor haemolysis (Tallqvist, HCS, Lovibond, …)

2. Techniques based on the red colour of blood after haemolysis of the red blood cells (DHT,…).

3. Techniques based on the transformation of the haemoglobin (Sahli, Hemocue, Drabkin, …).

The PCV (Packed cell volume) or hematocrit may also be used to screen for anaemia.

The choice between these techniques will be based on reliability, repeatability, precision, accuracy, price, equipment needed, level of technical difficulty, staff training level, …

REFERENCE VALUES[1]

The reference ranges for haemoglobin vary by age and sex as shown in the table below.

Age	Sex	Haemoglobin (g/100 ml)
3 months – 12 months	Men and Women	10,0 – 14,0
1 year – 12 years	Men and Women	10,5 – 15,0
12 years – 100 years	Men (Europe)	13,2 – 17,3
12 years – 100 years	Women (Europe)	11,7 – 15,5

[1] Reference ranges vary in different population and in different laboratories (different techniques). District laboratories should check the figures above for the technique in use with their nearest hematology reference laboratory.

HCS METHOD (HAEMOGLOBIN COLOUR SCALE)

PRINCIPLE :

The intensity of the red colour of blood corresponds with the amount of haemoglobin. The degree of anaemia can be visually assessed by matching the colour of a drop of blood on special filter paper against a standardized colour chart. The colour chart is developed to represent the colour range of normal to anaemic blood on filter paper : 14, 12, 10, 8, 6 and 4 g /100 ml.

EQUIPMENT AND SUPPLIES :

Blood collection equipment and supplies + Kit HCS (booklet of 6 shades of red, instructions for use, dispenser of 200 special absorbent test strips in handy box). Use only the special test-strips that are provided by Copack, since others may give inaccurate results. Keep these test-strips dry, clean and protected from direct sunlight at any time. 70 % Alcohol.

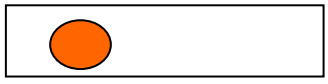
BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended.

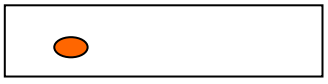
METHOD :

Find a suitable place: a room which is well-lit by daylight and/or artificial light. Avoid direct sunlight and marked shade. Do not read the scale in your own shadow.

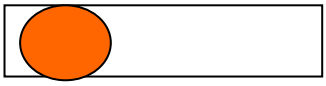
1. Place a drop of blood on one end of the test-strip so that it forms a spot, which is large enough to spread beyond the area of an aperture in the scale (about 1 cm in diameter).



Correct amount of blood : sufficient to spread beyond the area of an aperture in the scale.



Too little blood : The area of an aperture in the scale will not be covered.

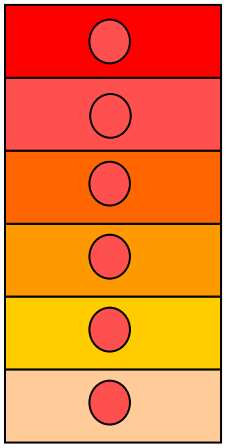


Too much blood : The spread will be too thick and the blood will take too long to dry.

2. Wait about 30 seconds after applying the blood; then read immediately. Any delay in reading the test will cause an error as the blood stain will change colour, becoming lighter and unreliable. Starting from the lightest shade or darkest shade, slide the blood stain up and down behind the apertures in the scale until you find the best colour match. When reading, keep the test-strip close to the back of the scale to prevent any stray of light from entering.

    - If the blood stain matches one of the shades of red exactly, record the haemoglobin value.
    - If the colour lies between two shades on the scale, record the mid-value.
    - If there is any doubt between two shades, record the lower value.

Example:



14 g/100 ml :Too light

12 g/100 ml: Correct.

10 g/100 ml: Too dark.

8 g/100 ml : Too dark.

6 g/100 ml : Too dark.

4 g/100 ml : Too dark.

MAINTENANCE :

To clean the scale, wipe the back side with a humid tissue (alcohol 70 %), then with a dry tissue. The scale should be cleaned at the end of each session and during the session if the surface becomes soiled during use.
The scale can be used for thousands of tests, but to avoid deterioration of the colours, always keep the booklet closed after use and never leave it exposed to direct sunlight. It should be replaced periodically.

LOVIBOND® METHOD (Harrison's method)

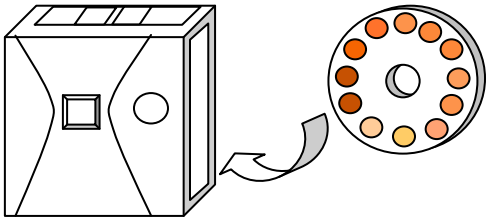
PRINCIPLE :

The Harrison's method is a simplified version of a visual comparative technique for estimating haemoglobin. The red colour of blood corresponds with the amount of haemoglobin. Blood is inserted directly into a special cell (thickness of 0,004") without any preliminary manipulation. The colour is compared with a series of reference glasses in a Lovibond® comparator.

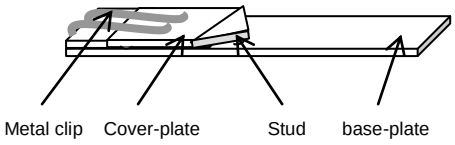
MATERIAL :

Blood collection equipment and supplies + Lovibond 2000 comparator, standard discs Lovibond 5/8 A and 5/8 B, Lovibond cell 0,004", soft paper, Beaker, chlorine solution 1 %, 70 % alcohol.

Lovibond® 2000 comparator Disc



Lovibond® 0,004"[2]



The blood cell consists of a base-plate and a cover-plate constructed from plain white glass. Fused on the cover-plate are three small studs of glass which create, when in position a cell of 0,004" thickness. This cell is filled from the side by capillarity.

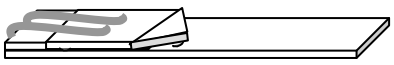
BLOOD COLLECTION :

Capillary blood. [For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended].

METHOD :

1. Clean and decontaminate the base-plate and the cover-plate, first with water, then with 70 % alcohol.

2. Place the two plates in position and join them with the clip.



[2] 0,004 inch = 0.1016 mm

3. Check if the discs are clean. Wipe with a cloth if they become dirty. Select the appropriate disc (evaluation of conjunctival pallor) and insert it into the comparator with the values towards the front of the instrument. Rotate the disc until the lowest or the highest value.

CHECK THE CELL BEFORE USING IT :

- Decontamination with hydrochloric solution, next with 70 % alcohol?
- Is it dry and dust free, and clean, no lines nor finger prints?
- Does the cover plate cover properly the base plate ?
- Are the figures 004 well readable?

4. Fill the cell by capillarity with capillary blood. If there are air bubbles in the cell, restart from point 1.

5. Clean the blood excess around the cell with a soft paper.

6. Remove the clip.

7. Place the cell, containing the prepared sample in the right compartment. Using a diffuse light (facing southern daylight in the southern hemisphere), rotate the disc until the closest colour matches with the sample. (Be quick to avoid drought or coagulation). The Lovibond value will be shown in the window in the bottom right corner of the comparator.

Find a suitable place for the colour comparison :

- During the day : Facing a white surface (southern daylight in the southern hemisphere).
- During the night : Facing a white surface illuminated by a white lamp (not fluorescent light).
- Direct sunlight or direct artificial light gives incorrect results.

8. Convert the Lovibond value in g/100 ml (cf. conversion table).

9. Decontaminate the Lovibond cell (contact with 1 % chlorine solution during 30 minutes).

10. To avoid deterioration of the colours, store immediately the disks in a box.

10. Clean and decontaminate the base-plate and the cover-plate, first with water, then with 70 % alcohol.
11. For practical reasons it can be advised to mount the plates in advance so that no time will be lost in case of emergency.

SMALL PROBLEMS AND SOLUTIONS :

ALWAYS CHECK THE CONCORDANCE BETWEEN THE HAEMOGLOBIN VALUE AND THE COLOUR OF THE CONJONCTIVES.

1. **No value appears in the inner opening of the comparator.**

   ⇒ The disk is placed back to front. Take out the disk and turn it round.

2. **No colour corresponds with the colour of the blood of the patient.**

   ⇒ The chosen disk does not correspond with the expected haemoglobin value. Use the other disk.

   ⇒ The patient is icteric (jaundice). His upper conjunctives are yellow. Find the closest colour and note the presence of jaundice in the report.

   ⇒ The blood colour is inferior to the minimum of the comparator. Give as result < than 20 % or < than 3,3 g/100 ml.

3. **The measured haemoglobin value seems to be too low.**

   ⇒ Beware that the cover plate is placed in the correct way (figures 004 readable). If this is not the case, restart the measurement with another plate.

⇒ Beware there are no air bubbles between base and cover plate. If this is not the case, restart the measurement with another plate.

⇒ Check the cleanliness of the comparison disk and, if necessary, clean it with a soft tissue.

⇒ Check the cleanliness of the translucent plate of the comparator and, if necessary clean it with a hydrochloric solution of 1 %, next with filtered water.

If none of these solutions are helping and if the values are systematically too low, make a comparison between the new and the old disks (the colour of the disks is degrading with light).

4. Too high haemoglobin values.

⇒ The upper side of the cover-plate and/or the under side of the base-plate are soiled with blood. Take of the blood on both sides with a soft paper.

⇒ The disinfectant used for the blood taking is stained (e.g.Betadine). Restart the measurement using Ethanol à 70 %.

⇒ Check the cleanliness of the translucent plate of the comparator and, if necessary clean it with a hydrochloric solution of 1 % , next with filtered water.

⇒ The patient is icteric (jaundice). His upper conjunctives are yellow. Find the closest colour and note the presence of jaundice in the report.

⇒ The patient is dehydrated (provoking a haemoconcentration). No solution, note the dehydration in the answer.

CONVERSION TABLE % LOVIBOND TO g / 100 ml :

IT IS HELPFUL TO TAKE THE COLOR MATCH UNTIL SLIGHTLY BELOW,
THEN SLIGHTLY ABOVE, IN ORDER TO FIND THE BEST COLOR MATCH.

DISKS	PERCENTAGE	g / 100 ml
Disc N° 5/8 A (light colour) For low Haemoglobin values	20	3.3
	24	4.0
	28	4.7
	32	5.3
	36	6.0
	40	6.7
	46	7.3
	52	8.7
	58	9.7
Disc N° 5/8 B (dark colour) For high Haemoglobin values	64	10.7
	70	11.7
	76	12.7
	84	14.0
	92	15.3
	100	16.7
	110	18.3
	120	20.0
	130	21.7

SAHLI METHOD

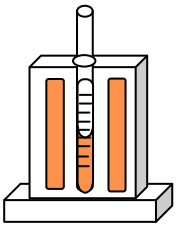
PRINCIPLE :

The Sahli method is based on converting haemoglobin to acid haematin (brown colour) and then visually matching its colour against a solid glass standard. Diluted hydrochloric acid is mixed into a graduated cylinder with an accurate volume of blood sample and distilled water is added until the colour of the diluted blood sample matches the glass standard. The dilution will be determined by the Haemoglobin level of the blood sample.

The Sahli method, still used in a lot of places, is not recommended. The Sahli method is not an accurate way of estimating haemoglobin: Not all the forms of haemoglobin are changed into acid haematin, the colour changes when viewed visually are not very great and the brown colour of the glass standard is not a true match for an acid haematin solution.

MATERIAL :

Blood collection equipment and supplies + Sahli haemoglobinometer, small glass rod, Sahli pipette, safety device for pipetting, Sahli tube and dropping pipette.



The Sahli haemoglobinometer is equipped with two glass colour standards. The Sahli tube graduated until 16 g/100 ml and/or in "percentage" (16g/100 ml = 100 %), is placed in-between.

REAGENTS :

Hydrochloric acid 0,1 N :

Hydrochloric acid, concentrated[☠]	:	9,5 ml
Distilled water	:	up to 1 litre

☠ **Caution, hydrochloric acid** is irritant and corrosive. Handle with care in well ventilated area (or in a fume cupboard). Fill until half a 1.000 ml volumetric flask with distilled water. Add slowly 9.5 ml concentrated hydrochloric acid. After cooling, fill until the 1,000 mark with distilled water and mix well. This reagent is stable for at least 1 year at room temperature.

Distilled water (or filtered water).

BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended.

METHOD :

1. Fill the graduated measuring tube up to the bottom graduation line with 0.1 N hydrochloric acid. (The mark level should be equal with the bottom of the meniscus formed by the liquid).

2. Check the tip of the Sahli pipette. Discard if broken (volume error). Check if the pipette is dry.

3. Draw the blood a little bit further than the 20 µl mark of the Sahli pipette. Do not allow air bubbles to enter. Wipe the outside of the pipette with absorbent paper and adjust the blood on the 20 µl mark.

4. Blow the blood from the pipette into the graduated tube of the acid solution.

5. Rinse the pipette by drawing in and blowing out the acid solution.

6. Allow to stand for 1 minute. The mixture will become dark brown and clear.

7. Place the graduated tube in the haemoglobinometer, compare the colour in diffused day light. Add water drop by drop and mix with the glass stirrer until the colour of the solution matches the colour of the reference tube.

8. When equal colours are reached, take the glass stirrer out of the graduated tube and read the level of the base of the menisci of the liquid. Hold the instrument about 50 cm away from your eyes, on the same height under diffuse light.

9. Note the reached mark that corresponds with the level on the tube.

DRABKIN METHOD

PRINCIPLE :

The haemoglobin cyanide method is the most accurate method of measuring haemoglobin and is considered as the reference standard (« gold standard"). Whole blood is precisely diluted 1 on 201 in a Drabkin solution. The red cells are haemolysed and the haemoglobin is oxidized by the ferricyanide to methaemoglobin. This is converted by the cyanide to stable haemoglobin cyanide.

$Hb + K_3Fe(CN)_6 \rightarrow MHb$

$MHb + KCN \rightarrow HiCN$

[Hb = haemoglobin, MHb = methaemoglobin, HiCN = haemiglobincyanide]

The chemical reaction takes place at a pH stabilized by a monopotassium phosphate buffer in order to obtain a complete reaction in a reasonable time. Addition of a detergent facilitates haemolysis and prevents turbidity caused by plasmatic proteins. The optical density is in proportion with the haemoglobin quantity that is present in the blood.
Absorbance of the HiCN is read in a spectrophotometer at the wavelength 540 nm or in a colorimeter using a yellow-green filter. The absorbance obtained is compared with that of a reference HiCN standard solution. Haemoglobin values are obtained in the calibration graph.

BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended. After thoroughly mixing with anticoagulant, the blood can be frozen for as long as 2 years (and used as control).

MATERIAL :

Blood collection equipment and supplies + spectrophotometer (or colorimeter) that transmits light at 540 nm, Sahli pipette (or 20 µl automatic pipette), 5 ml graduated pipette (or 5,0 ml dispenser), safety device for pipetting, test tubes, cuvettes, different volumetric flasks and different calibrated pipettes (for calibrator's preparation), graph paper.

REAGENTS :

Drabkin's neutral diluting fluid (pH 7,0 – 7,4) (also "ready to use" available example Sigma D 5941) :

Potassium hexacyano ferrate ($K_3Fe(CN)_6$)	:	0,20 g
Potassium Cyanide (KCN)☠	:	0,05 g
Potassium dihydrogen phosphate (KH_2PO_4)	:	0,14 g
Tween 20	:	1 ml
Distilled water	:	to 1 litre

(Tween20 can be replaced by 0.5 ml Brij-35 at 30 %)

☠ **CAUTION : Potassium cyanide is highly poisonous**. It is a pale yellow clear fluid which may no longer be used if its colour is lost or if it becomes turbid. The prepared Drabkin solution is stable for at least 6 months at room temperature protected from light (amber bottle).

Haemiglobin cyanide (cyanmethaemoglobin) standard for calibration:

HiCN reference standard solutions are stable for long periods and are commercially available as: Haemoglobin standard (SIGMA), HiCN BS 3985 (Merck/BDH), …

Example with Haemoglobin standard from SIGMA :

- Reconstitute one vial of the standard by adding 50,0 ml of the Drabkin solution.
- Mix well.
- Wait at least 30 minutes before use.

The standard must be stored at 2- 8°C in the dark and is stable for at least for 6 months.

Prepare a calibration graph from this HiCN reference standard. Plot the absorbance readings at 540 nm of different standard dilutions against their known concentrations of haemoglobin. The curve is linear, passing through the origin.

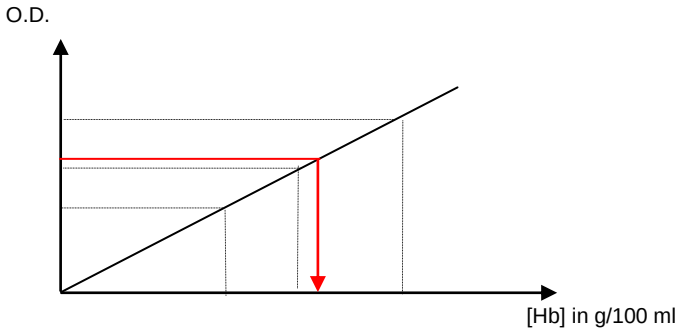
Dilution's example:

Tube N°	Volume in ml Drabkin solution	Volume in ml diluted standard	final concentration in g %
1	4	0	0
2	3.5	0.5	2.25
3	3	1	4.5
4	2	2	9
5	1	3	13.5
6	0	4	18

METHOD :

1. Set the spectrophotometer wavelength at 540 nm.
2. Set up a series of labelled test tubes for blank and tests.
3. Add 4.0 ml of the Drabkin solution to all tubes.
4. Add 20 µl of whole blood sample to each labelled test tube.
5. Rinse the pipette 3 times with the reagent.
6. Mix well and allow to stand for at least 5 minutes at room temperature. (Attention, the reaction can take until 30 minutes for a sample containing an increased proportion of carboxy haemoglobin).
7. Read and record absorbance of each test (or control) versus the blank as the reference at 540 nm in the same instrument used for preparing the calibration curve. The colour is stable for several hours.

Determine the haemoglobin concentration of each test directly from the calibration curve.



Or

$$\text{Calculated: } \frac{\text{O.D. test}}{\text{O.D. Calibrator}} \times \text{calibrator's concentration} = \text{sample's Hb concentration}$$

HEMOCUE® B

PRINCIPLE :

The HemoCue® is an example of a robust, portable and accurate haemoglobinometer readily available for use. Although not affordable by most district laboratories, it may be used for survey. The HemoCue uses calibrated disposable cuvettes that are treated with chemicals (sodium desoxycholate, sodium nitrite and sodium azide) which rupture the red cell wall and combine with the haemoglobin to form a compound (azidemethaemoglobin) which can be measured photometrically (modified Vanzetti reaction : Sodium nitrite converts the haemoglobin iron from the ferrous to the ferric state to form methaemoglobin. The methaemoglobin then combines with azide to form azidemethaemoglobin and is measured photometrically at two wavelengths, 570nm and 880nm). The result in g/100 ml is displayed in digital form on the face of the instrument. Web site : http://www.hemocue.co.uk/

MATERIAL :

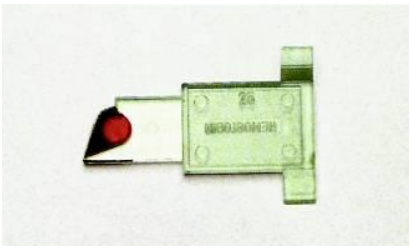
Blood collection equipments and supplies + HemoCue® B disposable cuvettes, HemoCue® B instrument, HemoCue® B standard control cuvette, batteries.

BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended.

METHOD :

1. Check the stability of the calibration : Place the control cuvette into the cuvette older and push it into the measuring position. The displayed value should not deviate more from the assigned value on the control cuvette card than + /- 0.3 g/100 ml (for our control cuvette N° 0149-003-037 the value lies between 11.0 and 11.6 g/100 ml).



2. Fill the disposable cuvette with the blood drop by touching the capillary tip of the cuvette with the blood drop.

3. Be sure that the cuvette is entirely filled with blood. If air bubbles are present, discard the cuvette and fill a new disposable cuvette.

4. Wipe off the excess of blood on the outside of the cuvette tip. Make sure that no blood is drawn out of the cuvette in this procedure.

5. Place the filled cuvette into the cuvette holder immediately and push it into the measuring position.

6. After approximately 30 to 50 seconds the result is displayed (in g/100 ml (or g/l or mmol/l). The filled cuvette should be analysed at least 10 minutes after it has been filled (evaporation).

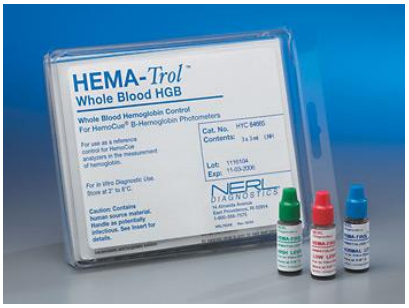
N.B. : 3 different models are now commercialized : HemoCue® B et HemoCue® 201+ for « normal working conditions » (temperature < 30°C and low humidity) and The Hb 301 system is optimized for use in primary care and designed for high temperatures and humidity. If the 2 first models (Band and 201) use the principle explained above, the model 301 uses a different analytical method : The measurement takes place in the analyzer, which measures the absorbance of whole blood at a Hb/HbO2 isobestic point. The analyzer measures at two wavelengths (506 and 880 nm) in order to compensate for turbidity. In fact, it's an automatised version of the Lovibond method.[3]

Hemocue® B

Hemocue® 201+

Hemocue® 301+

Microcuvettes B and 201 are to be stored at room temperature (15-30°C). The reagents contained within the HemoCue® microcuvettes B and 201 are moisture sensitive: Recap vial immediately after removing cuvettes and do not remove desiccant from the vial. The microcuvettes 301 are to be stored at 10–40 °C (50–104 °F). Once the seal of the vial is broken, the microcuvettes are stable for 3 months. An unopened vial of microcuvettes can be stored for a shorter period of time (6 weeks) between -18–50 °C. HemoCue Hb 301 Analyzer is only to be used with correspondent HemoCue Hb microcuvettes.



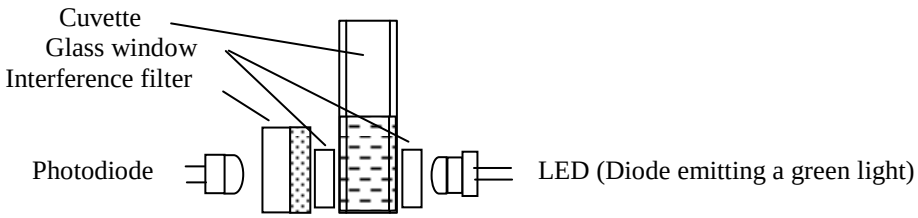
HemoCue® B-HB Photometer Hemoglobin Controls HYC84665 3x3 ml (1 low, 1 normal, 1 high) : HemoCue® B-HB Photometer Hemoglobin Controls. Features a 2-year expiration from date of manufacture at 2 – 8°C, 60-day open vial stability at 2 – 8°C, and a 30-day open vial stability at room temperature. Utilizes stabilized whole human red cells which process like a fresh patient sample.

[3] Evaluation of the utility of the Hemocue 301 haemoglobinometer for blood donor screening. L. D. Morris, A. Ossei-Bimpong, D. McKeown, D. Roper and S.M. Lewis. Vox sanguinis (2007) 93, 64-69.

DHT HAEMOGLOBINOMETER (Developing Health Technology) (Ammonia technique)

PRINCIPLE :

Whole blood is diluted 1 in 101 in a weak ammonia solution. The red cells are haemolysed and the amount of haemoglobin is measured over a narrow spectral band. The measurement of the optical density is carried out at a wavelength of 523 nm, the crossing point of absorption curves of various haemoglobin forms, which are thus detected with equal sensitivity. This optical density is automatically converted to read-out directly as haemoglobin concentration in g/l on a liquid crystal display. Zero and calibration need to be checked but are not adjustable (no calibrating solutions).



MATERIAL :

Blood collection equipment and supplies + DHT haemoglobinometer, test tubes, Sahli pipette (or 20 µl automatic pipette), 2 ml graduated pipette (or 2,0 ml dispenser), safety device for pipetting, 1.000 ml volumetric flask, 10 mm light path cuvettes.

BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended.

REAGENTS :

Ammonia solution 0,04 % (v /v):

Preparation with ammonia 28 % concentrated:

Ammonia 28 % concentrated	1,4 ml
Distilled water	up to 1.000,0 ml (volumetric flask)

CAUTION : Ammonia solution is a corrosive chemical with an irritating vapour. Handle with care in well-ventilated area (or in a fume cupboard). **Keep the stock bottle well stoppered.**

This solution is stable when kept in a tightly stoppered bottle. Renew every 4 weeks

Any concentration of any ammonia solution can be made by taking a volume of concentrated solution equivalent to the required % and making this up with distilled or deionised water to a volume equivalent to the % of concentration :

C1 x V1 = C2 x V2

C1 = Concentration of the concentrated ammonia solution
V1 = Volume of the concentrated ammonia solution

C2 = Needed ammonia concentration
V2 = Needed volume of the weak ammonia solution

Example : C1 = 28 %
V1 = ?
C2 = 0,04 %
V2 = 1.000 ml

28 % x V1= 0,04 % x 1000 ml ➔ V1 = 1,429 ml

METHOD :

It's vital to understand the factors that influence accuracy of measurement and dilution. The accuracy of haemoglobin measurement depends on accuracy of proportion of blood to diluent. The quality of the used water and ammonia as diluent is also quite important.

INITIAL CHECKS OF OPERATION AND FACTORY CALIBRATION : **(Caution : Used values are only valid for** the **haemoglobinometer N° 0931)** that is used at the ITM.

Do not touch the clear working side areas of the cuvette. Avoid contamination of these, handling the cuvette only by the top of the etched surfaces at front and back (non optical sides). Always ensure the outer surface of the cuvette is completely dry. When filling cuvette, ensure no air bubbles are present to affect measurement.

A. CHECK BLANK :

Install a CLEAN and DRY cuvette into the cuvette aperture. (Use the same kind of cuvette which is used for haemoglobin determination). The value on the display must correspond with the blank value BR1 (+/- 5) [14-24 g/l for our haemoglobinometer 0931]. If this is not the case check for the following cause:

PROBABLE CAUSE	**REQUIRED ACTION**
Cuvette not well installed :	Insert the cuvette with the mat etched surface in front of you. Check again the reading for BR1.
Scratched cuvette :	Use new cuvette. Check again the reading for BR1.
Dirty or wet cuvette surface :	Clean and dry the cuvette. Check again the reading for BR1.
Value of scale factor M incorrect :	Press and hold down the « R » button located at the back of the device. The symbol "HHH", and after some seconds the scale factor M will appear. Compare this value with the figure for M shown in "certification" (162 for our device). If there is a difference between these values, then adjust the displayed value by pressing the "L" button to lower the value and the "R" button to raise the value. Check again the reading for BR1.
Zero level of the device incorrect	Place a clean cuvette filled with distilled water into the cuvette window. Press and hold down the "L" button. At first the display will show the last reading, followed after several seconds by a "beep". After this beep the LCD turns off and the new zero level is stored in the device. Check again the reading for BR1.
Other type of cuvette used for the calibration :	Place a clean cuvette filled with distilled water into the cuvette window. Press and hold down the "L" button. At first the display will show the last reading, followed after several seconds by a "beep". After this beep the LCD turns off and the new zero level is stored in the device. Check again the reading for BR1.
Dirty or wet photocell windows :	Clean the glass of the photocell windows with alcohol on cotton swap stick. Check again the reading for BR1.

B. CHECK ZERO :

Place a cuvette containing 1-2 ml of the weak ammonia solution (0,04 %) into the cuvette aperture. (Use the same kind of cuvette which is used for haemoglobin determination). The value on the display should be zero. If this is not the case remove the cuvette and within 2 seconds press and hold the "L" button until the beep. Zero will automatically be reset. Check again the reading for zero.

C. CHECK CALIBRATION :

The permanent calibration control standard (checker), supplied with each instrument, is numbered and matched uniquely to one instrument. Place the checker into the cuvette aperture. The value of the displayed reading must correspond to the value CR1 (+/- 5) [133-143 g/l for our haemoglobinometer 0931]. If this is not the case check for the following causes:

PROBABLE CAUSE	REQUIRED ACTION
Cuvette not well installed :	Insert the cuvette with the plastic surface in front of you. Check again the reading for CR1.
Scratched control cuvette :	Buy a new cuvette. Check again the reading for CR1.
Dirty or wet control cuvette surface :	Clean and dry the cuvette. Check again the reading for CR1.
Value of scale factor M incorrect :	Press and hold down the « R » button located at the back of the device. The symbol "HHH", and after some seconds the scale factor M will appear. Compare this value with the figure for M shown in "certification" (162 for our device). If there is a difference between these values, then adjust the displayed value by pressing the "L" button to lower the value and the "R" button to raise the value. Check again the reading for BR1
Zero level of the device incorrect	Place a clean cuvette filled with distilled water into the cuvette window. Press and hold down the "L" button. At first the display will show the last reading, followed after several seconds by a "beep". After this beep the LCD turns off and the new zero level is stored in the device. Check again the reading for BR1.
Other type of cuvette used for the calibration :	Place a clean cuvette filled with distilled water into the cuvette window. Press and hold down the "L" button. At first the display will show the last reading, followed after several seconds by a "beep". After this beep the LCD turns off and the new zero level is stored in the device. Check again the reading for BR1.
Dirty or wet photocell windows :	Clean the glass of the photocell windows with alcohol on cotton swap stick. Check again the reading for BR1.

TEST METHOD :

Attention: The precision of the measurement depends for a great part on the skill and proficiency of the technician in preparing the right concentration and volume of ammonia and blood and of the quality of the distilled water.

1. Bring 2,0 ml of the ammonia reagent in a test tube for each sample to be measured
2. Check the pipette on its cleanliness, if it's dry and if the point is not broken.
3. Measure 20 µl of capillary blood or well-mixed venous blood
4. Aspirate the blood a bit higher than the 20 µl line of the pipette. Clean the blood on the outside of the pipette and adjust the volume with a clean absorbent paper.
5. Dispense this volume into the 2 ml of the ammonia diluting fluid by rinsing the pipette 3 times (aspirate and blow out the pipette).
6. Stop the tube and mix. The solution can be read immediately. The colour is stable for 6-8 hours.
7. Transfer the patient's sample to a clean 10 mm light-path cuvette.
8. Place the cuvette into the cuvette holder, wait for the audible signal and read the displayed haemoglobin value.
9. Return the sample to its tube and allow the cuvette to drain, e.g. invert it on a paper towel.

Caution, if the audible signal, accompanying the photometry process, ends before the cuvette is fully seated into the device, the result may be wrong. Wait a few seconds for the next measurement cycle to complete.

PACKED CELL VOLUME BY CENTRIFUGATION or HEMATOCRIT

PRINCIPLE :

The hematocrit level, or packed cell volume, is a measure of the ratio of red cells to the total volume of whole blood (plasma, white blood cells and red blood cells) and is expressed as a percentage.

$$PCV = \frac{\text{Volume of red blood cells}}{\text{Volume of whole blood}} \times 100 = x\ \%$$

In the new units, the PCV is expressed as a ratio (litre/litre), the same formula but without a multiplication by 100.

The blood is placed in a standard size capillary tube and centrifuged at high speed. After centrifugation, the volume occupied by the red cells is measured. Because of a uniform bore of the capillary, the length is directly proportional to the volume.

$$PCV = \frac{\text{Length of red cell column (mm)}}{\text{Length of total column (mm)}} \times 100 = x\ \%$$

EQUIPMENT AND SUPPLIES :

Blood collection equipments and supplies + micro hematocrit centrifuge (radius greater than 8 cm, able to achieve maximum speed within 30 seconds and to maintain a centrifugal force of at least 10.000*g* for 5 minutes without exceeding a temperature of 45°C) , Disposable heparinised capillary tubes (length 75 mm diameter 1.5 mm), spirit lamp (or clay sealant or plasticine), reference chart.

BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin is recommended. In this case, plain capillary tubes should be used. Since the hematocrit increases in function of the conservation time, the examination must be executed within 6 hours.

METHOD :

1. Fill about three quarters of either:

   A plain capillary with well mixed EDTA anticoagulated blood or
   A heparinised capillary with capillary blood.

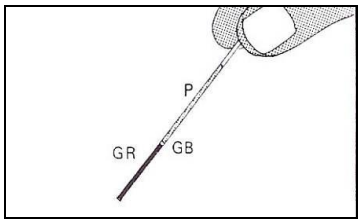
2. Seal by heat the unfilled end using a small flame from a spirit lamp (or seal the unfilled end using a sealant material).

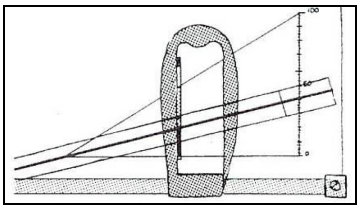
3. Carefully locate the filled capillary in one of the numbered slots of the micro hematocrit rotor with the sealed end against the rim gasket. Write the number of the slot on the patient's form.

4. Balance the diametrically opposite slot with a capillary and centrifuge at high speed for 5 minutes.

5. After centrifugation, the capillary tube will show 3 layers:

- At the top, a column of plasma (P).
- In the middle, a very thin white layer (Buffy coat) of white cells and platelets (GB).
- At the bottom, a column of red cells (GR).





Using a reference chart : Line up the bottom of the red cells at the zero mark. Slide the capillary tube along the scale until the top level of plasma reaches the 100 mark. The line passing through the top of the red cell column will indicate the packed cell volume. Do not include the buffy coat as part of the red cell level in the reading. After reading discard safety the capillary tube.

REMARKS :

Other information from the PCV : Plasma from normal blood appears straw-coloured. In iron deficiency it appears colourless. When it contains an increased amount of bilirubin, it will appear abnormally yellow. If the plasma is pink-red, this indicates a haemolysed sample (a new blood sample should be tested). In thalassaemia major, the red cells column appears dark-red. When white cell numbers are significantly increased (> 20.000/ mm^3), this will be reflected in an increase in the volume of the buffy coat layer. The microscopical examination of the dividing line between the white blood cells and the plasma, is used for microfilaria or trypanosome detection (Woo technique).

REFERENCE VALUES [4] :

In a similar way to haemoglobin levels, PCV values vary according to age, gender and altitude.

Age	PCV % (international units)
Children at birth	50 to 58 (0.50 to 0.58)
Children (3 months)	35 to 40 (0.35 to 0.40)
Children (1 year)	31 to 36 (0.31 to 0.36)
5 years	33 to 37 (0.33 to 0.37)
Adult women	36 to 45 (0.36 to 0.45)
Adult men	42 to 49 (0.42 to 0.49)

[4] Reference ranges vary in different population and in different laboratories (different techniques). District laboratories should check the above figures for the technique in use with their nearest hematology reference laboratory.

INTERPRETATION OF PCV:

PCV is an easy, simple but indirect technique to detect anaemia. It is of diagnostic value in patients suffering from anaemia, dehydration, shock or burns. The number of red blood cells, the size of red blood cells and plasma volume influence the PCV. If the number of red blood cell stays in the normal range, 1% PCV represents more or less 110.000 red blood cells/mm³ of blood.

As the ratio between the haemoglobin concentration in the red blood cells and the red blood cell volume is quite stable, there is normally a linear relationship between PCV and haemoglobin concentration. The formula {[PCV (in %) x 0,3] + 2} gives roughly an idea of the haemoglobin concentration. This is only true in case of normocytic or normochromic anaemia. This formula will not substitute the haemoglobin determination.

> Example : the estimation of the haemoglobin concentration for a PCV of 33 % is 10.1 g/100 ml [(33 x 0.3) + 0.2].

In case of a normocytic or normochromic anaemia, the formula stays equal, since hematocrit and haemoglobin are equally reduced.

> Example: For a hematocrit of 27 %, the haemoglobin is estimated on:
> (27 x 0.3) + 0.2 or 8.4 g/100 ml.

In case of a macrocytic or hypochromic or megaloblastic anaemia, but also in case of a microcytic anaemia, this estimation does not work.

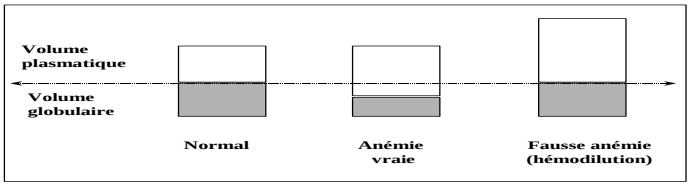


Fig.3 : True and “false” anaemia due to haemodilution.

PCV is decreased in anaemia. PCV values are increased in case of loss of plasma (dehydration, cholera, diarrhoea, severe burns, …), in dengue haemorrhagic fever, and (rarely) in all forms of polycythaemia.

SOURCES OF ERRORS :

Erroneously high PCV may be due to :

- Patient's position during the blood collection (10 % higher when standing compared to lying).
- Storing the specimen beyond 6-8 hours before performing the test.
- Delay of reading after centrifugation (plasma evaporation).
- Prolonged stasis caused by constriction with a tourniquet for more than 1 minute (haemoconcentration).
- …

Erroneously low PCV may be due to :

- Leakage from the tube during centrifugation due to insufficient sealing of the capillary tubes.
- Heparin degradation in hot climate (Tubes should be stored in a cool place).
- Dilution by interstitial fluid, especially where there has been difficulty in venous puncture or failure to obtain free flow of capillary blood
- Blood coagulation if the blood is not immediately mixed with anticoagulant.
- EDTA in excess of 2 mg/ml (diminution of the red cells volume).
- Secondary haemolysis to forcible passage through a fine bore needle.
- Haemolysis caused by heating of the blood, during the sealing of the capillary.
- Inadequate centrifugation (too short or with a too low centrifugal force.
- Blood not properly oxygenated: the blood must be sufficiently, but gently mixed before performing the test.
- Haemolysis of the sample during centrifugation by an overheated centrifuge.
- …

Erroneously high or low PCV may be due to :

- Using an inappropriate anticoagulant.
- Reading error (parallax error).
- Poor quality tubes which are not uniform bored.
- Inadequate mixing of the blood prior to filling the micro hematocrit tube.
- …

QUALITY CONTROL :

Specimens run in duplicate must agree within 3 %.

The complete packing of the red blood cells should be verified. After reading the PCV, re-centrifuge the tube for 2, 3 and 5 minutes more. No decreasing of the PCV should be found. In case of PCV value decreasing, choose the centrifugation time which give a constant PCV value.

RED BLOOD CELL INDICE (MCHC)

Red cell indices are frequently used in the investigation of anaemia. If a laboratory is able to measure a PCV and to perform an accurate haemoglobin determination, an MCHC (Mean Corpuscular Haemoglobin Concentration) can be calculated. The MCHC gives the concentration of haemoglobin in red blood cells.

MCHC (g/100 ml) = Haemoglobin concentration (g/100 ml) / PCV (%) x 100

Example : A patient with a haemoglobin of 16 g/100 ml and PCV de 45 %.
MCHC = 16/45 x 100 = 36 g %.

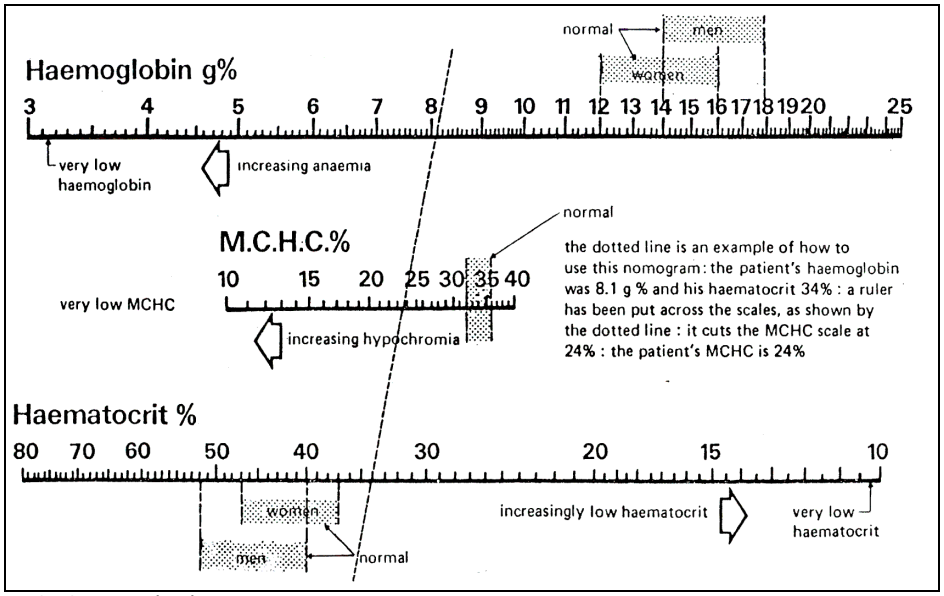


Fig. 4 : MCHC Determination.

A guideline reference range for MCHC in health is 30 - 36 g%. These figures should be checked locally. The MCHC is used in the classification of anaemia :

MCHC < 30 g%	**Hypochromic anaemia.**
MCHC between 30 and 36 g%	**Normochromic anaemia**.

Hyperchromic anaemia does not really exist : A red blood cell cannot contain more haemoglobin than the maximal continence (with one exception for the megaloblastic anaemia).

Hypochrome anaemia < 30 normochrome anaemia > 36 " *hyperchrome* anaemia "

CELL NUMBER CONCENTRATION

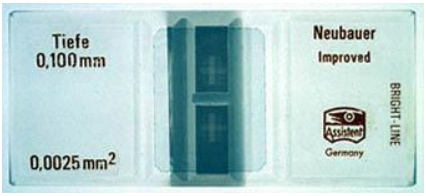
GENERAL PRINCIPLE :

The count of cells per volume unit is quite useful as diagnostic tool. Electronic counter systems are often not available (or affordable) in district laboratories. The only realistic alternative will be to use a counting chamber in which the cells are counted under the microscope. Depending on the kind of liquid to be analysed (expected cell number), a dilution and/or destruction of undesirable cells must be done. A simple calculation taking in account the volume in which the cells are counted and the dilution, will give the number of cells per mm^3 or per µl, in the initial biological liquid.

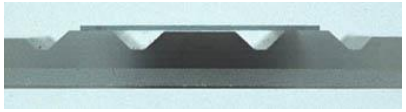
GENERAL MATERIALS :

Collection material + Sahli pipette (or automatic pipette), 1 ml graduated pipette, safety device for pipetting, test tubes, absorbent paper, diluting liquid, pencil, counting chamber, counting chamber cover glasses, plastic Pasteur pipette, hand tally counter, lens paper, microscope (objective 10 X and 40 x), chlorine solution.

COUNTING CHAMBERS :



C B A B C
Neubauer counting chamber



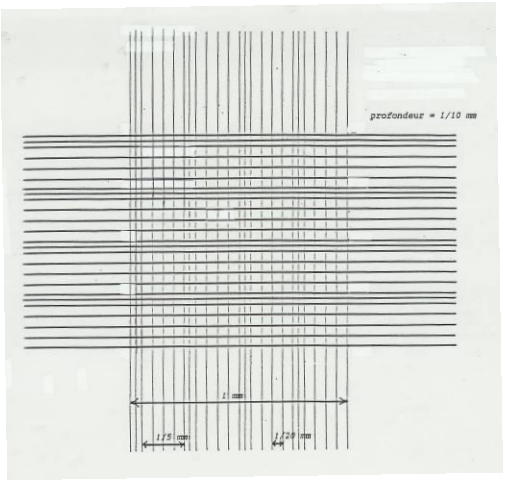
C B A B C
Neubauer counting chamber, sectional view

B A B
Neubauer counting chamber, sectional view

The upper side of the counting chamber is divided in 5 parts by moats or wells, (C, B, A, B and C). The central part A is also divided in 2 by a transversal channel. Each central part of the chamber contains a specially grid area with dimensions as shown in the figures (depending on the type of counting chamber). Counting chambers are so constructed that the distance between the underside of the cover glass and the surface of the chamber is constant (= depth of the chamber). The area and the depth of the counting will define a precise volume.

Characteristics of the most common counting chambers :

Small volume counting chamber :



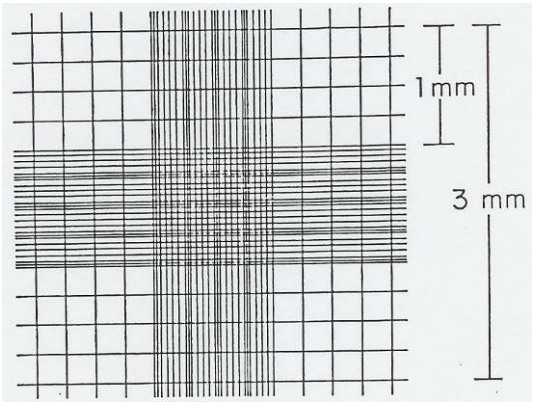
Thoma counting chamber:

Area : 1 mm x 1 mm : 1 mm².

Depth : 0,1 mm.

Total volume: 0,1 mm³ or µl.

Medium volume counting chambers :

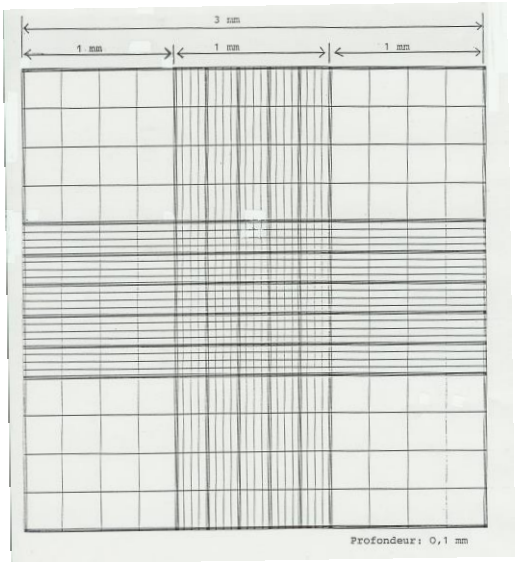


Neubauer counting chamber :

Area : 3 mm x 3 mm = 9 mm².

Depth : 0,1 mm.

Total volume: 0,9 mm³ or µl.

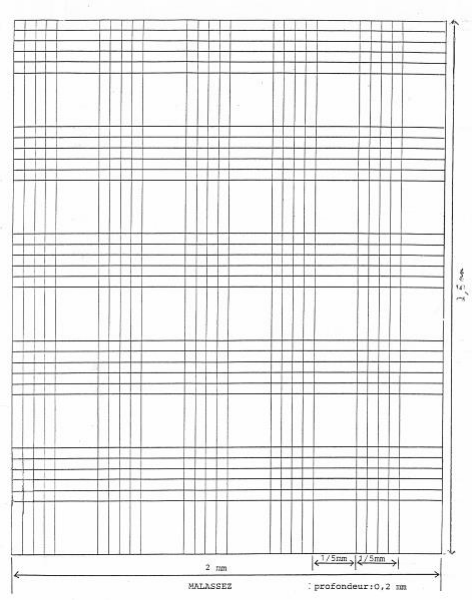


Neubauer (double improved) counting chamber:

Area : 3 mm x 3 mm = 9 mm².

Depth : 0,1 mm.

Total volume: 0,9 mm³ or µl.



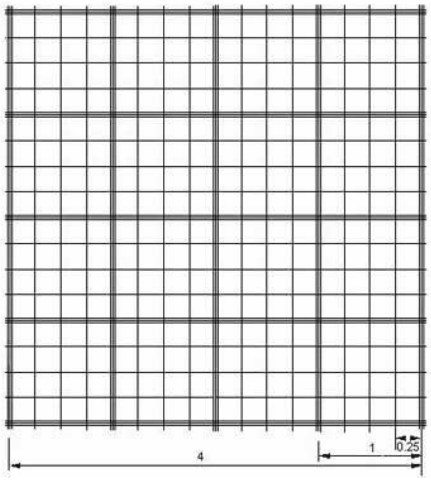
Malassez counting chamber :

Area : 2 mm x 2,5 mm = 5 mm².

Depth : 0,2 mm.

Total volume : 1 mm³ or µl.

Big volume counting chambers :

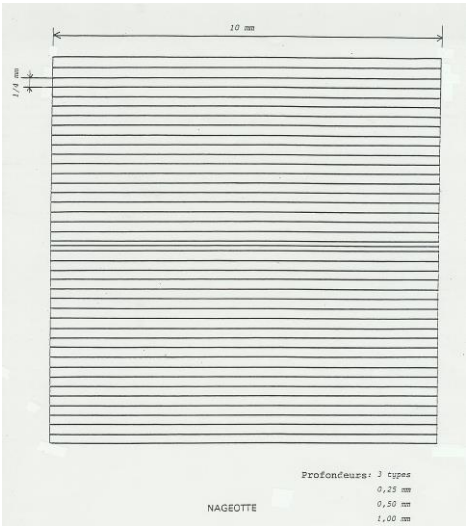


Fuchs-Rosenthal counting chamber :

Area : 4 mm x 4 mm = 16 mm².

Depth : 0.2 mm.

Total volume : 3,2 mm³ or µl.



Nageotte counting chamber:

Area : 10 mm x 10 mm = 100 mm².

Depth : 0,25 mm ;

0,50 mm ;

or 1mm.

Total volume : 25 mm³ or µl ;

50 mm³ or µl ;

100 mm³ or µl.

The used type of counting chamber is a matter of availability. Small volume counting chambers are better for liquids with a lot of cells (red blood cells count in blood for example). Big volume counting chambers are better for liquids with few cells (white blood cells in CSF for example). Medium volume counting chambers may be used for all kind of liquids. The Neubauer double improved counting chamber is the most common type.

WHITE CELL COUNT IN BLOOD

REAGENT :

Turck solution :

Fill a bottle with 96 ml of distilled water
Add 3 ml concentrated (glacial) acetic acid (CH_3COOH) and mix
Add 1 ml gentian violet 1 % (w/v).

- **Caution: acetic acid** is a corrosive chemical with an irritating vapour. Handle with care in well ventilated area (or in a fume cupboard). **Never pour water in pure acetic acid.** Addition of a small quantity of water in acid produces enough heath to cause an explosion of the bottle.
- This reagent is stable for at least 3 months in a fridge.

BLOOD COLLECTION :

Capillary or venous blood. For venous blood, dry anticoagulant should be used (to avoid dilution). EDTA di-potassium salt or heparin are recommended.

METHOD :

1. Check the tip of the Sahli pipette. Discard if broken (volume error). Check if the pipette is clean and dry.

2. Pipette 0,38 ml of Turck solution into a test tube, using the 1 ml graduated pipette.

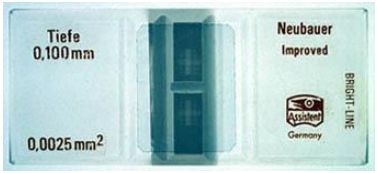
3. Label the tube with the patient's name and/or number. Draw venous or capillary blood at the 0.020 mark of Sahli pipette. Do not allow air bubbles to enter.

4. Wipe the outside of the pipette with absorbent paper. Check that the blood is still on the 0.02 ml mark.

5. Expel the blood into the test tube. Rinse the pipette by drawing in and discharging the fluid from the test tube 3 times. The dilution of blood is 1 on 20.

6. Mix the diluted blood well and wait 3 minutes before filling the counting chamber (red blood cells lysis).

7. Assemble the counting chamber : Prior moistening of the chamber surface on each side of the grid areas (« b »), is necessary for the cover glass to adhere to the chamber. Slide the cover glass into position over the grid areas and press down on each side until rainbow colour (Newton's rings) are seen.

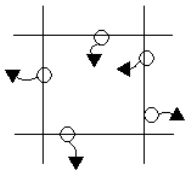


C B A B C
Neubauer counting chamber

8. Mix the diluted blood well. Use a Pasteur pipette (or a Sahli pipette) to fill the counting chamber completely. Take care not to overfill beyond the ruled area. **Caution** : if the liquid overflows into the channel between the two chambers, you must start again : remove and clean the coverslip, clean the counting chamber and refill with another drop.

9. Leave the counting chamber on the bench for 3 minutes to allow the cells to settle.

10. Place the counting chamber on the microscope stage.



11. Using the 10 x objective with the condenser iris sufficiently closed to give good contrast, focus the rulings of the chamber. Then, using the 40 x objective, count the leukocytes in the four large corner squares of the chamber which have a surface of 1 mm². Leukocytes appear as small, transparent cells, with light blue nucleus. Do not take dust or unlysed red cells for leukocytes. Include the cells, lying on the lines of two sides of each square in the count (use all the time the same lines) and exclude the cells on the two other sides.

12. Calculate the number of leukocytes in 1 mm³ of blood by multiplying the number of leukocytes counted in the four large squares by 50. Explanation of calculation :

    - **Dilution factor** : 20 µl of blood + 380 µl Turck solution, gives a **20** x blood dilution [(20+380)/20].
    - **Volume factor** : 4 large squares counted, or 4 x 0.1 mm³. Thus division by 4 and multiplication by 10 will give the number of leukocytes in 1 mm³ of diluted blood [10/4 = **2,5**].

    Global factor 50 [20 x 2,5].

Example :

Large square n° 1	25 leukocytes
Large square n° 2	26 leukocytes
Large square n° 3	24 leukocytes
Large square n° 4	26 leukocytes
Total for 4 larges squares :	**101 leukocytes**

Number of leukocytes par mm³ (or µl) of blood = 101 x 50 = 5.050

REFERENCE RANGES:

Normal leukocyte number concentration by age group (per mm³ of blood) [5]								
AGE	**1 day**	**15 days**	**2 months**	**6 months**	**2 years**	**6 years**	**12 years**	**Adults**
Number	9.000 to 30.000	6.000 to 20.000	5.500 to 18.800	6.000 to 17.500	6.000 to 17.000	5.000 to 14.500	5.000 to 13.500	4.000 to 11.000

QUALITY ASSURANCE :

For patient samples done in duplicate, the difference between the two counts should not be more than 20 %. For statistical reasons, the precision of the measurement will decrease with the number of counted cells. To decrease the errors for low counts, it may be good to repeat the count, using a lower dilution or counting the cells in more than 4 large squares. (Caution, in these cases, the calculation should be adapted)

It can be good to compare the distribution of the cells in the 4 large squares from the Neubauer chamber. The number of cells, counted in each of the 4 squares should not differ by more than 10 %.

[5] Cf. also addendum 2. References ranges vary in different population and in different laboratories (different techniques). District laboratories should check the above figures for the technique in use with their nearest hematology reference laboratory.

SOURCES OF ERRORS :

- Position of the patient during the blood collection [haemoconcentration].
- Nucleated red cells may cause an erroneously high count of WBC. This can be corrected by determining the proportion of nucleated red cells to white cells on a blood film.
- Prolonged stasis caused by constriction with a tourniquet for more than 1 minute [haemoconcentration].
- Cells counting from a dehydrated patient [heamoconcentration].
- Secondary haemolysis to forcible passage through a fine bore needle.
- Storing the specimen beyond 6-8 hours before performing the test.
- Blood collection from an arm in which an intravenous infusion is being given [haemodilution].
- Using an inappropriate anticoagulant.
- Sample coagulation.
- Disintegration of leukocytes when specimen stands. Specimen must be counted within 6 hours of collection.
- When using anticoagulated blood, not mixing the blood sufficiently or not checking the sample for clots.
- Counting chamber or cover glass dirty.
- Incorrect measurement of blood or dilution fluid due to poor technique or using a wet or chipped pipette.
- Dilution fluid contaminated with dust particles.
- Inadequate mixing of blood with diluting fluid.
- Insufficient lyses of RBC may create problems in identification of leucocytes.
- Air bubbles in the counting chamber or in the pipette [volume error].
- Inappropriate covering of the counting chamber [volume error].
- Over-filling a counting chamber or counting cells when sample contains air-bubbles.
- Counting chamber is not sufficiently filled.
- Not allowing sufficient time for the cells to settle in the chamber.
- Use of a too intense light source or not reducing the iris diaphragm to give good contrast.
- Calculation error.
- Administrative error.
- …

RED CELL COUNT IN BLOOD :

The same kind of technique can be used for the red blood cell count. Unfortunately, the precision is poor and it is not recommended for clinical practice. To calculate the MCV and the MCM indices, an accurate red blood cells count is needed. This needs an electronic cell analyser. Most district laboratories will not therefore be able to calculate these indices. However, examining a well–stained blood film can help to detect macrocytosis or microcytosis.

RED CELL INDICES

MCV
Mean Corpuscular Volume

$$MCV = \frac{\text{PCV (\%)}}{\text{Red blood cell count per µl (in millions)}} \times 10 \times 10^{-15}\ \text{l}$$

Reference range : 82 to 92 fl (for adults. Age related variation for children) [a femtolitre (fl) is 10^{-15} of a litre]

FOR ADULTS
microcytic anaemia < 82 normocytic anaemia > 92 macrocytic anaemia

MCH
Mean Corpuscular Haemoglobin

$$MCH = \frac{\text{Haemoglobin (in g/l)}}{\text{Red blood cell count per µl (in millions)}}$$

Reference range: 28 to 32 pg (for adults ; Age related variation for children)

FOR ADULTS
> 32 “hyperchromic anaemia”

ADDENDUM 1 : TECHNIQUES FOR ASSESSING ANAEMIA (PRICE LIST)

HCS:

- Starter kit containing: 1 Cover-box with 1 dispenser and 200 test-strips, 1 Booklet with Colour scale, 1 instruction manual, 4 refill dispenser with each 200 tests strips
- Price: 21.80 € (Feb. 2004, Copack).
- Refill kit containing:
- 10 dispenser boxes with each 200 test-strips (total 2000 tests)
- Price 32.75 € (Feb. 2004, Copack).

MICROHAEMATOCRIT CENTRIFUGE:

- Centrifuge DHT 590: 832.25 € (Feb. 2004, DHT)
- Centrifuge Transfer : 1500 € (Feb. 2004, Transfer)
- Capillary tubes heparinised 75 mm (200 tubes): 4.80 € (Feb 2004, DHT)
- Sealing paste for capillary tubes (6): 4€ (Feb 2004, DHT)

LOVIBOND HAEMOGLOBINOMETER:

- Comparator Lovibond 2000 : 456.22 € (Jan 2004, Transfer)
- Special capillary chamber for Lovibond : 53.33 € (Jan 2004, Transfer)
- Colour standard disc 5/8 A lower haemoglobin : 90.05 € (Jan 2004, Transfer)
- Colour standard disc 5/8 B higher haemoglobin : 161.98 € (Jan 2004, Transfer)

DRABKIN colorimetric determination:

- Colorimeter WPA C0700D, wavelength range 400-700 nm: 734 € (Feb 2004, DHT)
- Total Haemoglobin kit sigma 525-A 1000 determination, with haemoglobin standard: 80.74 € (Dec 2003) **TRANSPORT AND STORAGE 2-8°C.**
- Sahli pipette: 1.34 € (Feb 2004, DHT)
- Graduated glass pipette 5 ml: 1.93 € (Feb 2004,VWR) or dispenser 4 ml Ceramus 217 € (Feb 2004, VWR).
- Devices for pipetting : 2.92 € (Feb 2004, VWR)
- Optical cuvettes 10 mm light path (100 cuvettes): 4.58 € (Feb 2004,DHT)
- + Bottles
- + Test tubes
- + Test tubes rack

HEMOCUE:

- Hemocue® B photometer with control cuvette: 555 € (Feb 2004, Hemocue)
- Disposable micro cuvettes Hemocue ® B (50 cuvettes): 60 € (Feb 2004, Hemocue)
- Hemocue® 201 photometer with control cuvette: 327,80 € (July 2006, MSF Supply)
- Disposable micro cuvettes Hemocue ® 201 (200 cuvettes): 84,66 € (March 2007, MSF Supply)
- Hemocue® 301 photometer with control cuvette: 350,00 € (July 2007, Hemocue)
- Disposable micro cuvettes Hemocue ® 301 (200 cuvettes): 75 € (July 2007, Hemocue)
- Batteries 4 (Type N alkaline cells) : 6.35 € (Feb 2004, DHT)

DHT HAEMOGLOBIN METER:

- Haemoglobin meter DHT HB 523: 543 € (Feb 2004, DHT)
- Ammonia 30 % 1 litre: 5.35 € (Feb 2004, VWR) **! Corrosive and irritant !**
- Sahli pipette: 1.34 € (Feb 2004, DHT)
- Graduated glass pipette 2 ml: 1.93 € (Feb 2004,VWR) or dispenser 2 ml Ceramus 217 € (Feb 2004, VWR).
- Devices for pipetting 2.92 € (Feb 2004, VWR)
- Optical cuvettes 10 mm light path (100 cuvettes): 4.58 € (Feb 2004,DHT)
- Batteries 4 (Type N alkaline cells) : 6.35 € (Feb 2004, DHT)
- + Bottles
- + Test tubes
- + Test tubes rack

SAHLI:

- Sahli kit : 57.35 € (Feb 2004, VWR)
- Hydrochloric acid 37 % : 9.35 € (Feb 2004, VWR) **! Corrosive and irritant !**

GENERAL :

- Blood lancet
- Disinfectant (Sodium hypochlorite or chlorine-releasing disinfectants)
- Cotton
- Alcohol 70 %
- …

ADDENDUM 2 : SOME HAEMATOLOGICAL REFERENCE RANGES (GUIDELINE FIGURES)[6]

Age	Sex	Haemoglobin	RBC	PCV	MCHC	MCV	WBC
Units		g/100 ml	per µl x 10^6	%	g/100 ml	fl	per µl x 10^3
3 – 12 months	Male	10,0 – 14,0	3,0 – 5,6	26 – 41	28,4 – 40,0	70 - 105	5,5 – 17,5
3 – 12 months	Female	10,0 – 14,0	3,0 – 5,6	26 – 41	28,4 – 40,0	70 – 105	5,5 – 17,5
1 – 12 years	Male	10,5 – 15,0	3,8 – 5,5	32 – 42	32,0 – 37,0	72 - 94	5,0 – 14,0
1 – 12 years	Female	10,5 – 15,0	3,8 – 5,5	32 – 42	32,0 – 37,0	72 – 94	5,0 – 14,0
12 – 100 years	Male (Europe)	13,2 – 17,3	4,3 – 5,7	39 – 49	32,0 – 36,0	81 - 100	4,0 – 11,0
12 – 100 years	Female (Europe)	11,7 – 15,5	3,8 – 5,1	35 – 45	31,5 – 36,0	81 – 100	4,0 – 11,0

N.B. : Man and women (Asia) : 4.000 – 10.000 leukocytes /µl
Man and women (Africa) : 2.600 – 8.300 leucocytes /µl

Differential WBC reference range :

CELL TYPE	Percentage (absolute number)	
Polymorphonuclear	**EUROPE + ASIA**	**AFRICA**
Basophils	0 – 1 % (0 - 200)	0 – 1 % (0 - 200)
Eosinophils	0 – 4 % (0 - 400)	0 – 5 % (0 - 500)
Neutrophils (non segmented)	0 – 5 % (0 - 700)	0 – 5 % (0 - 700)
Neutrophils (segmented)	50 – 75 % (1.800 - 7.000)	30 – 40 % (900 - 4.000)
Monomorphonuclear		
Lymphocytes	30 – 40 % (1.000 - 4.000)	40 – 60 % (1.200 - 6.000)
Monocytes	0 – 8 % (0 - 800)	0 – 8 % (0 - 800)

Reticulocyte count :

Percentage : Adults and children : 2 - 15 / 1.000 RBC
Infants at birth : 20 - 60 / 1.000 RBC

Absolute number : Adults and children : 25.000 - 160.000 / µl
Infants at birth : up to 150.000 /µl

Platelet count : 150.000 - 400.000 / µl

[6] References ranges vary in different population and in different laboratories (different techniques). District laboratories should check the above figures for the technique in use with their nearest haematology reference laboratory.

ADDENDUM 3 : MORPHOLOGY OF BLOOD CELLS IN A MAY-GRÜNWALD-GIEMSA STAINED BLOOD FILM

CELL TYPE	SIZE	NUCLEUS			CYTOPLASM		
GRANULOCYTES	µm	FORM	COLOR	CHROMATIN STRUCTURE	QUANTITY	COLOR	GRANULES
LEUKOCYTES : POLYMORPHONUCLEAR GRANULOCYTES							
IMMATURE NON SEGMENTED NEUTROPHILS "Band forms" or "S" shaped	12 – 15	Horseshoe, Central curvature is maximum a third part of the width of the lobes [7]	Clear blue purple	Strands of fine chromatin	abundant +++	Dusty rose (=very small granules)	small granules, light purple or violet Not always present
SEGMENTED NEUTROPHILS	12 – 15	2 to 5 lobes [8]	Deep blue purple	Rather thick and coarse	+++	Rose	Small granules, Pink or pink mauve
EOSINOPHILS	12– 15	Usually a bi-lobed nucleus	Blue purple	Rather thick and coarse	+++	Rose	Many large, uniform granules, red orange
BASOPHILS	11 – 13	Hardly visible lobes, not well separated (polymorph)	Blue purple	Rather thick and coarse, covered by granules	+++	Light rose	Very large, well separated, variable granules Deep purple Small in number

[7] **Left deviation of the Arneth formula: an increase over 16 % of non segmented neutrophils, yet immature forms, occurring in inflammations, but also in stress conditions,… 2 to 5 segmented neutrophils are the major fraction of the neutrophils in a normal leukocyte type.**

[8] **Right deviation of the Arneth formula: in contrast with the left deviation, where segmented cells are rarely seen, this image shows hyper segmented neutrophils, with 5 or more lobes. A hyper segmentation is characteristic for megaloblastic anaemia. In the early phase, more than one neutrophil with 6 lobes per 100 granulocytes is found.**

CELL TYPE	SIZE	NUCLEUS			CYTOPLASM		
AGRANULOCYTES	µm	FORM	COLOR	CHROMATIN STRUCTURE	QUANTITY	COLOR	GRANULES
LEUKOCYTES : MONOMORPHONUCLEAR AGRANULOCYTES							
SMALL LYMPHOCYTES	7 -10	Round or oval Or slightly indented	Deep purple	Big clumps of intensely stained chromatin	(-) or +	Sky blue (Often absent)	
LARGE LYMPHOCYTES	10 – 15	Round or oval Or slightly indented	Red, purple	Clumps of deep stained chromatin and other clumps which are less intensely stained	++	Sky blue	Absent or a few granules azurophils (rose violet)
MONOCYTES	15 – 25	Round, oval, indented or bean form	Blue to slightly violet	Fine, spongy like	+++ Vacuoles often demonstrable.	Grey or blue grey	Very fine granules (dusty like), azurophils (rose violet)
ERYTHROCYTES	6,7 – 7,7				Biconcave discus shape	rose	none
TROMBOCYTES	1,5 – 2 (5)				Slightly blue		Reddish

Instituut voor Tropische Geneeskunde
Institut de Médecine Tropicale
Institute of Tropical Medicine
Instituto de Medicina Tropical

Nationalestraat, 155
B – 2000 Antwerpen

Stichting van Openbaar Nut 0410.057.701

POSTGRADUATE IN TROPICAL MEDICINE AND INTERNATIONAL HEALTH
MODULE 1
CLINICAL & BIOMEDICAL SCIENCES OF TROPICAL DISEASES

BLOOD TRANSFUSION

(In remote areas)

Practical notes

SEPTEMBER 2017

Philippe Gillet – Luc Boel - Jan Jacobs
jjacobs@itg.be

SHORT SUMMARY OF BASIC GENETICS

The genetic information required for cell life is stored in the DNA. Human cells contain a total of 23 pairs of chromosomes (diploid number). A **chromosome** is the concentration form of the chromatin (DNA + histones) which becomes visible during cell division (mitosis or meiosis).

During the development of male and female reproductive cells, a special type of cell division occurs: the meiosis. This reduces the number of chromosomes in the spermatozoid or the ovum to half the number (haploid number) found in normal body cells. So, when the ovum is fertilized by one spermatozoid, the zygote which results contains the full diploid number of chromosomes (46, 23 from the father and 23 from the mother). The chromosomes from a same pair are **homologous**.

Genes are the small units strung along the length of chromosomes. A **gene** is the factor at a particular point or **locus** on the chromosome which represents a hereditary characteristic. Alternative or slightly contrasting forms of the gene are known as **alleles**. Alleles are generally represented by a character, in capital for the dominant allele and in small character for the recessive allele).

When the locus on a pair of homologous chromosomes is occupied by the same allele, the person is homozygous for the particular gene characteristic (**homozygote ZZ or zz**). If however, the alleles are different, the person is said to be heterozygous for the particular gene characteristic (**heterozygote for instance Zz**).

The alleles inherited for any particular characteristic can be dominant, co-dominant or recessive. A **dominant** allele will always show itself if it is present, whereas a **recessive** allele will only show itself if there is no dominant allele. If both alleles are expressed together, they are **co-dominant**. A recessive allele is manifested only in the homozygote.

The genetic composition for a particular inherited characteristic is called the **genotype** (gene composition) and its manifestation, or biological effect, is called the **phenotype** (gene expression).

SHORT SUMMARY OF BASIC IMMUNOLOGY

Immune reactions, used or involved, in the blood group determination, in post transfusion reactions or compatibility tests are mostly humoral immune responses.

Principal characteristics of the humoral immune response:

1. Essential contact with an antigen.
2. Immune tolerance. The capacity of the organism to make it unresponsive to "foreign" or "self" antigens.
3. Specific response of the antibody against the antigen.
4. Immunogenicity: The capacity to induce the formation of antibodies is different for different antigens (Antigenic power).
5. A step in the antibody response. Production of different antibody classes.
6. Memory, difference in primary and secondary response.

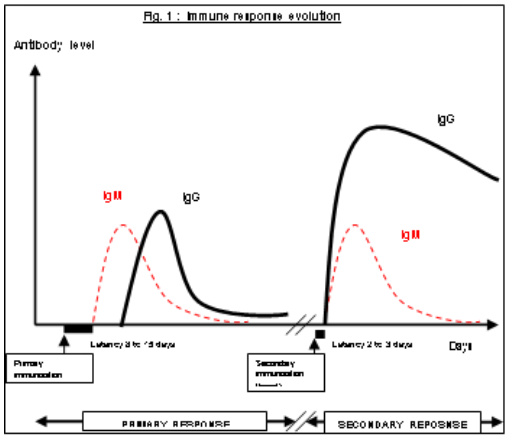


Fig. 1 : Immune response evolution

BLOOD GROUPING

A blood group is a group of individuals who has a (allotypical) character in common which distinguishes them from other groups. This characteristic, which is carried by the blood elements, has an antigenic activity. However, by language restriction, the expression « blood groups » is only used for the red blood cell groups, but there exist also blood groups for platelets, for polynuclear WBC, lymphocytes and proteins.

The antigens of the blood groups are located on the membrane of the red blood cells (either exclusively, either also on other types of cells). They can be proteins, but most of the time they consists of glucides (sugar) complexes: Glycoproteins, glycolipids etc. They are essentially known by their antigenic capacity; their biological functions are often hardly known: Transporters and membranous channels (proteins assuring the molecule transport through the membrane), of enzymes, of structural proteins of the membrane (« skeleton » of the cell), of adhesion molecules, or of membranous receptors (proteins capable to link with a signal or informative molecule, …). The immunology of blood groups is essentially circulating (immunity with antibody and complement interaction). Their study must thus consider the corresponding antigens and antibodies.

The classification is made on two levels:

1. The first level consists of all the **antigens** of blood groups. Up to now over 650 groups are described. (Examples: Ag A, Ag B, Ag D, Ag Fy^a, …).

2. All these antigens are grouped in a second level in **systems**. A blood group system is the total of antigens developed by the alleles of the same genetic mono factorial unit. A system is composed of the total of antigenic variants of membranous components.

Antigens are thus immunologically defined, while the systems have a genetic definition.

29 blood systems are described at this moment. [International Society of Blood transfusion]. (Examples ABO, Rhesus, Duffy, Kell, Lewis, P, Diego, Lutheran, Chido/Rodgers, …)

In the **current minimal transfusion practice**, only the two most important systems are taken into account:

- The **ABO system** that represents the major obstacle in all transfusions by the presence of natural antibodies (it is also a system of tissular histocompatibility antigens HLA).
- And the **Rhesus system**, since the D antigen is the most immunogenic of all the blood group antigens.

The determination of blood groups is following the "**4 x 2 rule**[9]**"**:

- **Two technicians.**
- **Two series of different reagents from different producers, using different techniques (in tubes, on slides, in gel, …).**
- **Two different techniques. (test and counter test, or forward and reverse blood grouping)**
- **Two samples taken at different moments.**

This rule of "4 x 2" permits to give a definite card of blood group.

In the practice of a small laboratory these "4 x 2" rule is not applicable. Without giving a blood group card, it is possible to realise transfusions relatively sure, based upon one determination, by one person on one sample. But then the compatibility test or at least the « rapid cross match » must be absolutely performed. (Verification of the ABO compatibility)

[9] **République Française, Ministère de la santé, de la famille et des personnes handicapées : CIRCULAIRE DGS/DHOS/AFSSAPS N° 03/ 582 of 15 december 2003** relative à la réalisation de l'acte transfusionnel et **CIRCULAIRE DGS/SQ 3 N° 99/14 du 12 janvier 1999** relative au respect de la réglementation en vigueur pour la détermination des groups sanguins ABO.

A THE ABO BLOOD GROUP SYSTEM

The ABO blood groups system was the first to be discovered. This was attributed to Karl Landsteiner In 1901. For this observation, which he described as the ABO (0) blood group system, Landsteiner was awarded the Nobel Prize for medicine (1930).
The ABO system presents an important characteristic that is at the same time at the origin of techniques of blood grouping and that explains also its crucial role in blood transfusion : the constant presence of antibodies anti-A and anti-B corresponding with the absent antigens on red blood cells of the subject.
The ABO antigens are terminal **sugars** of complex membrane macromolecules. The addition of these sugars is coded by a gene. This gene bears 3 alleles:

1. O : inactive enzyme (no added sugar).
2. A : N-Acetyl galactosamine transferase enzyme.
3. B : Galactose transferase enzyme.

The transmission of the ABO groups follows the laws of Mendel :

A and B are **dominant** over O
A and B are **co dominant**
O is relatively **recessive** to A and B

The ABO Antibodies are principally natural antibodies, complete, cold (optimally at 4°C, but they still agglutinate at 37°C), of **IgM** type. They appear spontaneously during young childhood by cross antigenic stimulation with surface antigens of saprophytic bacteria of the intestinal flora. They appear usually between the 3rd and the 6th month of life and their concentration reaches a maximum at the age of 10 years. They are present on every individual who does not possess the corresponding antigen on his own red blood cells.

Summary ABO blood group system

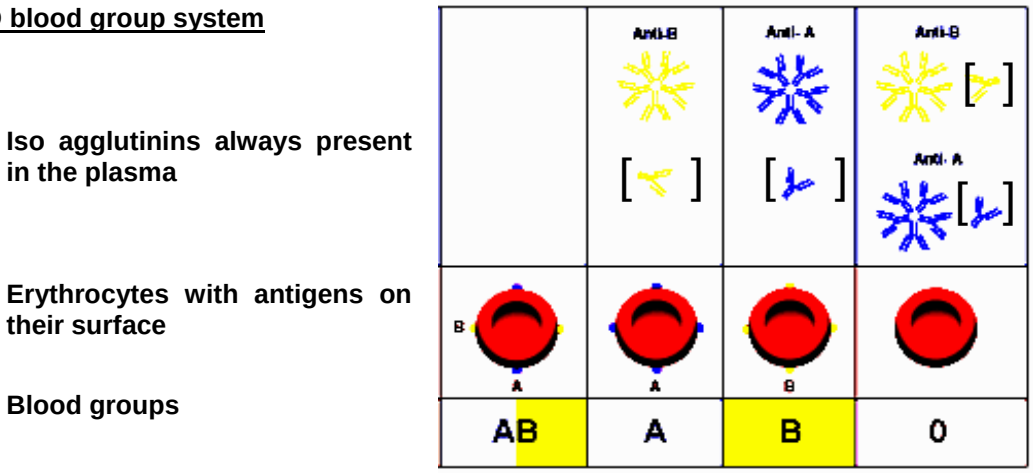


Table 1 : Estimation of the frequency and function of the skin colour:

ABO blood group	AB	A	B	O
Frequency of occurrence of ABO groups (related to skin colour) [10]				
White	4 %	44 %	9 %	43 %
Yellow	13 %	28 %	23 %	36 %
Black	4 %	27 %	21 %	48 %

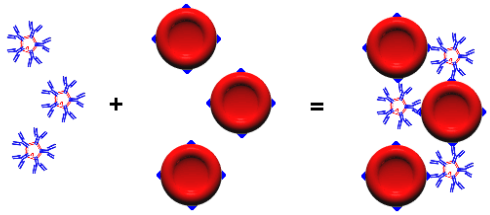
N.B. 1 : **Subgroups A_1 and A_2** : Antigen A exists as strongly reacting antigen A_1, and a weakly reacting antigen A_2. Most people who are group A or AB posses A_1 antigen, but up to 20 % belong to the subgroup A_2 or A_2B
Very soon a first complexity level has been reported concerning the phenotype A : The phenotype A_1 is characterised by the presence of the A_1 antigen while the phenotype A_2 does not possess the antigen A_1. The number of antigenic sites in phenotype A_1 subjects is much higher (1.000.000) than in subjects of phenotype A_2 (about 200.000 per red blood cell). This is resulting in weaker intensity reactions for phenotypes A_2 than for phenotypes A_1 (this explains the importance of the quality of the used reagents in the blood grouping). The practical distinction between these two phenotypes is of no importance in the transfusion context. One can although observe sometimes anti-A_1 or irregular natural anti-H, but this concerns most often of cold antibodies of a low titre, without any consequences in the transfusion aspect. Other rare phenotypes have also been described (phenotypes cis-AB, phenotype B(A) and A(B), acquired phenotype A, …). Other variants of group A (A_3, A_{erd}, A_{bantu}, A_x, etc.) and more exceptionally of group B weakly reacting have been reported.

[10] Approximate average percentages. Marked differences can occur between ethnic groups.

N.B. 2 : Occasionally, **IgG** hyper immune anti-A or Anti-B can be found in the serum of group O persons in response to stimulation by A and/or B like substances present in the environment, following pregnancy immunization or following the injection of some vaccines (immune irregular antibodies). Serious haemolytic reactions can occur when Group O whole blood containing anti-A and/or anti-B haemolysins is used to transfuse non group O persons (cf. dangerous O page 59). As IgG anti-A and/or anti-B can cross the placenta, there are also involved in some haemolytic disease of newborn.

Technique for ABO grouping on card (Beth-Vincent method or forward ABO grouping)

The determination of the ABO group reposes on demonstration of red blood cell surface antigens. Therefore known sera, directed against these antigens, are used. These serums agglutinate the red blood cells possessing antigens against which they are directed. There also exists the inverse determination (reverse blood grouping), which demonstrates antibodies that are present in the serum: known erythrocytes are then used. The serum assay is used as a confirmation of the red blood cell assay.



Example: Serum **Anti-A** + Red blood cells **A** = Agglutination

Samples:

Receptor blood obtained by capillary sampling (or venous blood on EDTA).

Reagents:

Anti-A, anti-B, [anti-AB] humans Diamed (Diaclon) for slide method.
Blood group cart (or on a glass slide or on an opaline plate), timer.

Technique: **The manufacture's instructions must be followed[11].**

1. Put on a card 2 [or 3] drops of receptor blood.
2. Near to each blood drop, depose a drop of each anti serum (anti-A, anti-B, [anti-AB]).
3. Mix the blood with the anti serum with the bottom of a tube.
4. Tilt the slide during 1 minute.
5. Read and note the result of the agglutinations immediately.

Anti-A	Anti-B	Anti-AB	Group ABO
+++	-	+++	A
-	+++	+++	B
-	-	-	O
+++	+++	+++	AB

+++ = agglutination (mostly very strong), - = absence of agglutination. Agglutination indicates the presence of the corresponding surface antigen on the red blood cells.

Possible problems:

False negative reactions:

- Immature antigens A and B (newborns).
- Genetic variants: Weak groups A or B, ...
- Antiserum of bad quality (antibody titre too weak).

False positive reactions:

- Coagulation of the blood to be determined
- Presence of cold agglutinins in the tested blood.
- Bacterial contamination of the test reagents
- Chronical infection (rouleaux formation by increased plasmatic proteins).
- Infection of trypanosomiasis (presence of auto agglutinins and rouleaux formation)
- Antigenic modifications during malign pathologies

[11] This technique is only applicable for the human Diacon antisera (Diamed). The technique may vary in function of the used reagents. Always follow the particular instructions indicated in the note of the company. Verify if the antisera may be used for the determination on slide.

B THE RHESUS SYSTEM

The discovery of the Rhesus system, as well as numerous other systems of red blood cell groups has been the result of the exploration of transfusion incidents and of haemolytic diseases of neonates.

The attribution of the name « Rhesus » to this system has its origin in a historical confusion with another system: In fact in 1939, Levine and Stetson concluded that the serum of a woman who delivered a baby attacked by a neo-natal haemolytic disease, agglutinating not only the erythrocytes of the child and the father, but also these of 85 % of the tested subjects. Landsteiner and Wiener in 1940 found that the diluted serum of a rabbit immunised with erythrocytes of *Macacus rhesus* agglutinated the erythrocytes in the same subjects. In fact, taking into account that the non diluted serum of the rabbit recognised 100% of subjects, it seemed that these hetero antibodies recognised a different antigen than the D antigen, being present on the majority of human erythrocytes, but from which the antigenic density is more important in subjects bearing the D antigen than in subjects which are deprived of it. The involved antigen in this confusion was named LW (Landsteiner and Wiener) and the term Rhesus has been maintained to design the initially concerned system.

The Rhesus antigens are **membrane proteins**. The addition of these proteins is coded by two genes. One gene coded for the D, one gene coding for the Cc and Ee. It is a very polymorph system: Nowadays 52 antigens are described (Within these, the DCcEe antigens are the most immunogenic). Its nomenclature, somewhat careless, reposes on 3 genetic hypotheses, the base of 3 theories, of which each is used according the circumstances: It is described as DCE (conception of Fisher and Race), it is named « Rh » (concept of Wiener), and it is exposed in figures (concept of Rosenfield).

For the **current minimal transfusion practice,** only the D antigen is important. It is own to human and to red blood cells. It is the most immunogenic of the blood group systems.

A subject possessing the D antigen on the surface of his red blood cells is called Rhesus positive (D + or Rh +)

A subject who does not possess the D antigen is Rhesus negative (D - or Rh – or d)

This system does not possess natural antibodies: So a « normal » person does not possess any anti Rhesus antibodies in his plasma. They appear by immunisation as a consequence of blood transfusions or by pregnancy. The Rhesus antibodies are immune antibodies, warm, of the **IgG** type, incomplete (non agglutinating in saline solution).

Table 2 Geographical distribution of the D antigen :

Percentages of Rhesus positive persons [12]	
South-East Asia and the Pacific	98 to 100 %
Equator and Chilli	91 to 97 %
Brazil and Argentina	82 to 94 %
Africa (Bantus, Ethiopians)	94 to 97 %
Africa (others)	82 to 94 %
Western Europe and North America	80 to 85 %

[12] Approximate average percentages. Marked differences can occur between ethnic groups.

Technique for the Rhesus grouping on slide (restricted to antigen D)

The determination of the Rhesus group rests on the detection of surface antigens of red blood cells. Therefore known serum is used, directed against these D antigens. This antiserum agglutinates red blood cells which possess antigen D. **For the slide technique the D antiserum must be of the IgM type.**

Samples:

Receptor blood obtained by capillary sampling (or venous blood on EDTA).

Reagents:

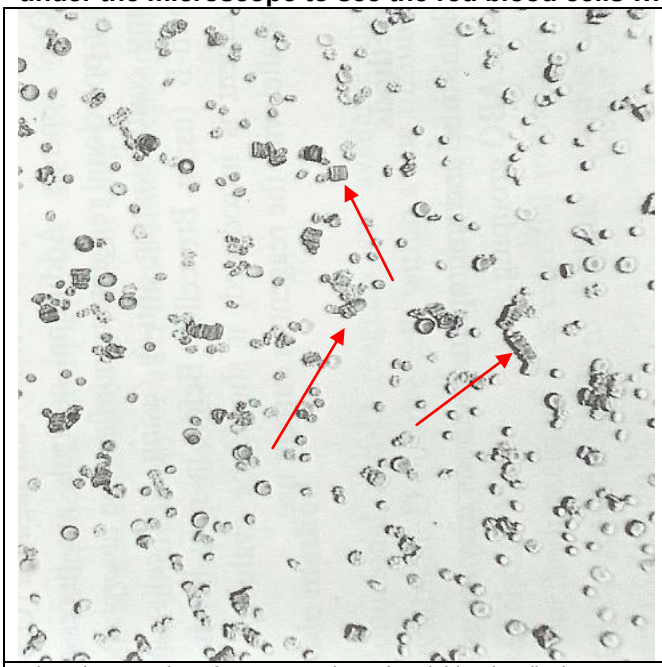
Monoclonal Anti-D Diaclon of Diamed (for slide method).
Blood group cart (or on a glass slide or on an opaline plate), timer, (spirit lamp).

Technique[13]:

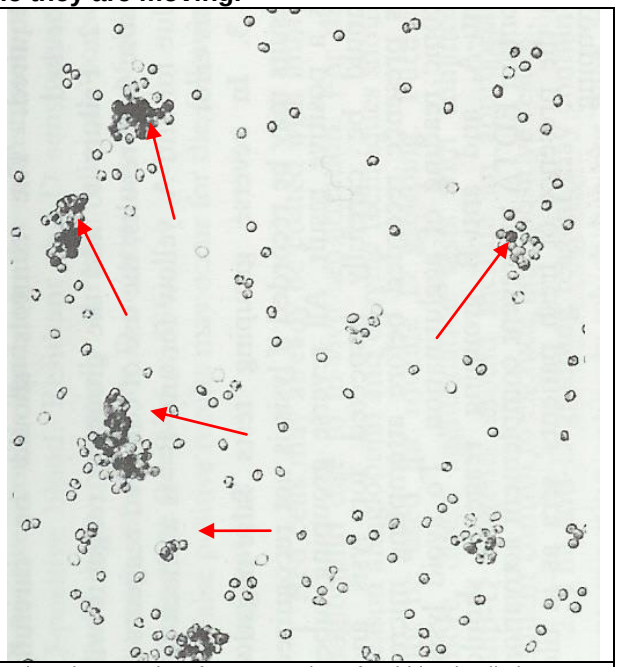
1. [Preheat a glass slide to 37°C.] The quality of the actual antisera is such that heating is only necessary if the test is negative.
2. Put on the glass slide (or on an opaline plate) 1 drop of blood to be tested.
3. Put a drop of anti-D near to the drop of blood.
4. Mix the blood and the anti-serum with the bottom of a tube.
5. Tilt the slide during 1 minute.
6. Read and note the result of the agglutinations immediately.

A positive reaction (presence of agglutination) means Rhesus positive. Absence of agglutination indicates a Rhesus negative result.

In case of doubt, observe the slide under the microscope (magnification 100x) to distinguish better the agglutinations. In order to make lecture easier, incline slightly the slide before lecture under the microscope to see the red blood cells while they are moving.



Microphotography of a suspension of red blood cells in serum, presenting a weak proportion of **rouleaux** (red blood cells on a pile of plates). Magnification 100x. [***N.B.** : Rouleaux formation is related to plasmatic proteins concentration].*



Microphotography of a suspension of red blood cells in serum showing a **weak agglutination** (red blood cells in small clusters). Magnification 100x.

[13] This technique is only applicable for monoclonal Diacon (Diamed anti-D antiserum). The technique may vary in function of the used reagent. The manufacture's instructions must always be followed. Check if the antiserum may be used for a reaction on slide and if it contains IgM.

Possible problems in the Rhesus grouping:

False negative reactions:

- Weak D (weak expression of the antigen)
- Partial D (D^u).
- Quality of anti-D. (antibody titre to low, or IgG and not IgM
- …

False positive reactions

- Coagulation of the blood to be determined
- Presence of cold agglutinins in the blood to be determined.
- Bacterial contamination of the test reagents
- Chronical infection (rouleaux phenomena by increased plasmatic proteins).
- Antigenic modifications during malign pathology
- Infection of trypanosomiasis (presence of auto agglutinins and rouleaux formation)
- …

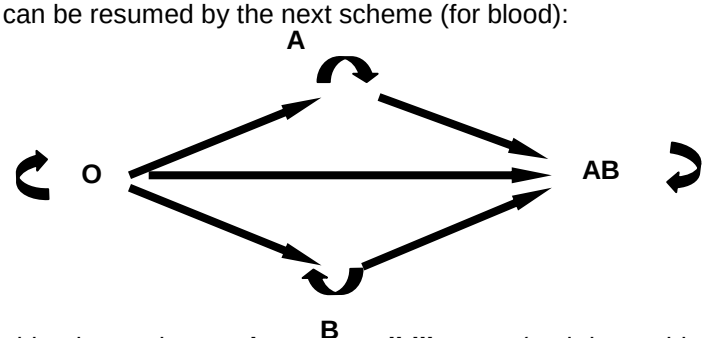
TRANSFUSIONAL RULES

1. Avoid Antigen – Antibody conflicts

Two types of antigen - antibody conflicts can be distinguished: The major and the minor conflicts. The important difference between these two is based upon the quantity and concentration of the involved antibodies.

Major conflict:

The blood of the receptor may not possess antibodies directed to the antigens of the red blood cells of the donor (Principle defined by Ottemberg in 1911). This compatibility in the ABO system is an essential condition for transfusions. Based on this principle, the blood compatibility can be resumed by the next scheme (for blood):



Moreover the blood grouping, **major compatibility** test (and the rapid cross match) permits to detect the presence of this type of antibodies.

Minor conflict:

It is also important to avoid to transfuse blood (especially when it is complete blood) containing antibodies directed against red blood cells of the receptor. This problem is greatly avoided by transfusing in iso-groups. To determine the presence of other antibodies, a test **of minor compatibility** can also be executed.

2. Avoid as much as possible the production of antibodies

Another important remark in blood transfusion is to avoid introduction of an antigen (especially when it is very immunogenic) which the receptor does not possess (Principe *non nocere*). . In fact, this introduction will bring along the production of antibodies which may have dramatical consequences for future transfusions (or for future pregnancies).

Summary for transfusions on district hospital level (For whole blood, with ABO and Rhesus grouping)

→ Execute as much as possible transfusions in iso groups ABO and if impossible, follow table 3 on next page.

→ A Rhesus negative person must be transfused with Rhesus negative blood.

Table 3: Order of choice in the selection of a blood donor (for whole blood, based upon the ABO group and upon the determination of the D antigen of the Rhesus system):

Blood group of the receptor	Preferred group of the donor	Order of choice in case that no iso group nor iso-Rhesus blood can be found
A Rh +	A Rh +	A Rhesus –, O Rhesus +, O Rhesus –
A Rh –	A Rh –	O Rhesus –
B Rhesus +	B Rhesus +	B Rhesus –, O Rhesus +, O Rhesus –
B Rhesus –	B Rhesus –	O Rhesus –
AB Rhesus +	AB Rhesus +	AB Rhesus –, A Rhesus +, A Rhesus –, B Rhesus +, B Rhesus –, O Rhesus +, O Rhesus –
AB Rhesus –	AB Rhesus –	A Rhesus –, B Rhesus –, O Rhesus –
O Rhesus +	O Rhesus +	O Rhesus –
O Rhesus –	O Rhesus –	/

N.B. : In **transfusion situations of full blood not in iso-groups**, the present IgM anti-A or anti B cause mostly only **small problems** because they are in insufficient quantity for provoking important haemolysis during a standard transfusion. (In all cases of complete blood transfusion, it is as well recommended not to mix the blood pocket to reduce the quantity of transfused plasma by sedimentation). Nevertheless a situation of haemolytic accident risk exists when blood is transfused from a donor presenting IgG anti-A or, more rarely anti-B. This type of rare haemolysin occurs mainly in donors of group O and appears by commutation from IgM anti-A or anti-B (cf. N.B.2 of ABO group). The haemolytic potential of an IgG is much more important than these of IgM which explains that these accidents happen with very low quantities of transfused haemolysin during a standard transfusion (this is even true for concentred cells !).
Dangerous universal donors are individuals of group O presenting a haemolysin of the ABO system. Their blood must be reserved for iso-groups transfusions (thus for an O receptor). Since a small laboratory doesn't have the possibility to detect the dangerous O donors, it is very important to privilege as much as possible transfusions in iso-groups. A basic technique permitting to detect **a part** of dangerous O is although described on page 23. This difficult operational technique is rarely used on district level, since dangerous O donors are rare and the preferred iso-group policy.

COMPATIBLITY TESTS

Moreover the ABO antibodies (mostly natural and regular), one can find other antibodies directed against **non ABO** erythrocyte antigens. Generally they are irregular immune antibodies which take particular techniques to be demonstrated. Their presence in the blood of an individual is mostly due to an immunisation against one or more antigens during a **preceding blood transfusion** or in women during **pregnancy**. The risks depend on the immunogenecity of antigens: by range of importance in the Rhesus system D, E, c,e,C ; the K of the Kell system; the Fy^a of the Duffy system; the Jk^a of the Kidd system,… To do well, all these antigens must be taken into account (or at least the most immunogenic antigens) before executing a transfusion. This is obviously impossible, even for a well equipped laboratory. In isolated situations, where only the ABO groups and the D of the Rhesus antigen are determined, the approach of the haemolysin (compatibility tests) is therefore most important.

The aim of the compatibility tests is to prevent an immunological transfusion reaction by demonstrating the incompatibility between donor and receptor. It permits thus to assure to the receptor the benefit of a transfusion with reduced immunological risk. In case of a positive compatibility test (presence of haemolysin), the search for compatible blood will not be easy : Without knowing neither the antigen or the concerning antigens, neither the principal donor's blood group, finding compatible blood will just depend on the perseverance in the search of a donor and in a great part of luck.

In this notes only the « rapid cross match» and the major compatibility in saline medium, associated with the indirect Coombs in LISS albumin medium, will be practiced. The majority of the considered techniques in a district laboratory are resumed in table 5 (page 19) and 6 (page 20). In this table, the most important advantages, but also the most important inconveniences are considered for each technique.

Rapid test (rapid cross match)

To avoid confusion errors of the patient, it is recommended to do an ultimate bed side control of the ill person in order to detect the ABO errors. In basic laboratory conditions, this minimal compatibility test can be performed in the laboratory as a "compatibility test". Nevertheless it cannot replace the major compatibility test (detection of IgM and IgG). **It is only useful for transfusions in iso-groups (presence of antibodies anti-A and anti-B in the blood of a person of group O). This test has thus only a restricted capacity, since it will only detect, almost exclusively, an ABO grouping error (restricted detection of IgM) in the context of an iso-group transfusion.**

Reagents:

Alcohol 70°.

Material:

Lancets, slides, needles.

Technique:

1. Verify the identity of the patient to be transfused. If he is able to, confirm this information by the patient, loudly spoken.
2. Verify if the blood group of the patient matches the blood group of the pocket.
3. Prepare an object slide.
4. Disinfect with alcohol 70° the tubing of the blood pocket and the arm of the patient.
5. Prick by means of a sterile lancet the finger of the receptor
6. Put a drop of blood of the receptor on the slide.
7. Prick by means of a needle the tubing of the blood pocked (!!! In order to avoid contamination of the blood pocket, there must be pricked between two knots or between two weldings).
8. Put a blood drop of the tubing on the slide.
9. Mix both blood drops with the corner of another slide.
10. Move and tilt during at least one minute.
11. Watch if any agglutination appears.

If agglutination is observed: ABO incompatibility or presence of antibodies directed against red blood cells of IgM type. DO NOT TRANSFUSE THE BLOOD BAG. Check the blood group of the donor and receptor.

If absence of agglutination: The blood bag can be transfused.

In case of doubt, observe the slide under the microscope (magnification 100x) to distinguish better the agglutinations. In order to make lecture easier, incline slightly the slide before lecture under the microscope to see the red blood cells while they are moving. (cf. microphotography page 8 for interpretation).

<u>Possible problems in the « rapid cross match »</u>

False negative reactions:

- Too weak red blood cell concentration, resulting in a difficult lecture (p.e. a very anaemic patient, with not much red blood cells).
- Too short reaction time.
- …

False positive reactions:

- Coagulation of the blood to be determined.
- Presence of cold agglutinins in the blood to be determined.
- Chronical infection in the donor or the receptor (rouleaux phenomena by increased plasmatic proteins).
- Infection of trypanosomiasis (presence of auto agglutinins and rouleaux formation).
- …

More complete tests

Complete Major compatibility test in saline medium
followed by an indirect Coombs test[14] in LISS-Albumin medium.

Antibody examination of the receptor versus erythrocytes of the donor. (IgM in Saline medium and IgG in LISS-Albumin).

Sample:

Serum of the receptor.
Red blood cells of the donor (blood taken on anticoagulant).

Reagents:

- Polyvalent Coombs Serum directed against human IgG and the fractions C3 of the complement. Diaclon Coombs serum (Diamed).
- LISS Diamed medium (DiaLISS - albumin). [LISS = Low Ionic Strength Solution]
- Physiological water 0.9 % (p/v) in NaCl (= saline or saline solution).

Sodium chlorite (NaCl).....	9 g
Distilled water.....	1000 ml

CONSERVATION : a few months.
CONDITIONS : Brown **or** white flask of 1000 ml.
Label : physiological water (or saline solution) and note the date of preparation.

Material:

Haemolysis tubes in plastic, 10mm x 75mm,
Plastic Pasteur pipettes,
Bulb pipettes for physiological water,
Haematological centrifuge,
Vacuum pump,
Water bath 37°C,
Microscope mirror [slides, microscope].

Technique [15] :

1. Take a sample (+/- 1 ml) of red blood cells of the donor in a haemolysis tube.
2. Wash the red blood cells 3 times with physiological water.
3. Dilute the red blood cells to 5 % in physiological water (50 µl of the pellet of the washed red blood cells + 950 µl physiological water). Homogenise the tube well.
4. Take 2 haemolysis tubes:

[14] The principle of the reaction is explained on page 52.
[15] This technique is only applicable for Diaclon Coombs antiserum (Diamed), associated with DiaLISS-albumin medium (Diamed). The technique may vary in function of the used reagents. Always follow the particular instructions which are indicated in the user manual of the producer.

TUBE 1

Saline medium

2 drops of red blood cells of the donor 3 times washed and diluted at 5 % in physiological water.
2 drops of serum of the receptor.

Incubate 5 minutes at room T° (22°C).

Centrifuge 1 minute at 1.000 RPM (100*g*).

Read and evaluate the result.

[a test is positive if there is presence of agglutination or haemolysis (total or partial) of red blood cells]

Demonstration of antibodies (complete of IgM type) :

ABO Error
Cold allo antibodies
Cold auto antibodies

ACTIONS (CF. MORE DETAILED EXPLANATIONS ON PAGE 51)

EXCLUDE THE ABO ERROR BEFORE TRANSFUSION:

Verify ABO group of donor
Verify ABO group of receptor
Verify if false positive
(Perform an auto-test with the receptor)

TUBE 2

Coombs indirect LISS albumin Medium

2 drops of red blood cells of the donor 3 times washed and diluted at 5 % in physiological water
2 drops of serum of the receptor.
4 drops of LISS (DiaLISS-Albumin).

Incubate 5 minutes at 37°C. **(sensibilisation step)**

Wash three times in physiological water; pour off well the supernatant after the last wash.

Add 2 drops of Coombs polyvalent serum. **(demonstration step)**

Centrifuge 1 minute at 1.000 RPM (100*g*).

Read and evaluate the result.

[a test is positive if there is presence of agglutination or haemolysis (total or partial) of red blood cells]

Demonstration of antibodies (incomplete of IgG type) :

Warm allo antibodies
Warm auto antibodies
False positives

ACTIONS

Verify if false positive
This always concerns dangerous antibodies

DO NOT TRANSFUSE, FIND ANOTHER DONOR

PROBLEMS AND INTERPRETATION OF THE MAJOR COMPATIBILITY TESTS

False negative reactions (in saline medium and/or in LISS albumin medium) :

- Incorrect cell concentration involving a difficult lecture.
- Incorrect wash of the red blood cells resulting in an inhibition of the Coombs serum.
- Unclean material bringing along an inhibition of the Coombs serum.
- Quality of the Coombs serum (expired, specificity, activity, titre, …).
- Incorrect concentration of the reagents (Coombs or LISS-albumin).
- Temperature and/or incubation time incorrect.
- …

POSITIVE REACTION IN SALINE MEDIUM :

- **False positives**
  - Presence of fibrin or bacterial contamination of the serum
  - …

1 Haemolysis :

- Concentration of NaCl incorrect of physiological water
- Centrifugation too fast,
- …

2 Rouleaux><agglutination : check agglutination under a microscope (see microphotograpy page 8 for interpretation):

- Chronical infections (rouleaux formation caused by plasmatic proteins increase).
- Trypanosomiasis infection (presence of auto agglutinins and rouleaux formation).
- …

- **ABO error** : Do not transfuse, verify the blood group of the donor and receptor and find a compatible donor

Execute an auto test (see page 17) [Not so useful in a district laboratory].

Exclusion of ABO error and exclusion of false positives, with positive auto-test : presence **of cold agglutinins** (cold agglutinins are cold auto antibodies which are active between 4 and 22°C). Practically, blood may be transfused at 37°C (except if the indirect Coombs is positive).

Exclusion of ABO error and exclusion of false positives, with negative auto-test : Presence of **cold allo antibodies** (these cold allo antibodies are not dangerous from the point of view of transfusion on condition that they are not active at 37°C. Practically, blood may be transfused at 37°C (except if the indirect Coombs in LISS-albumin medium is positive).

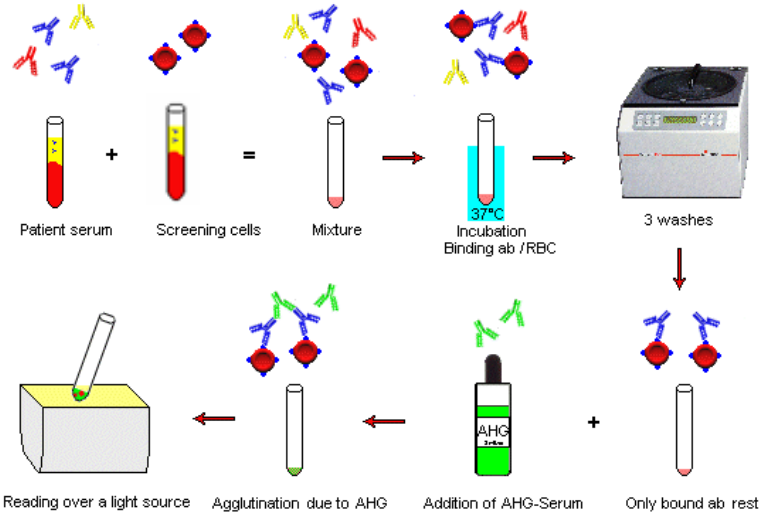
POSITIVE REACTION IN INDIRECT COOMBS IN LISS ALBUMIN MEDIUM :

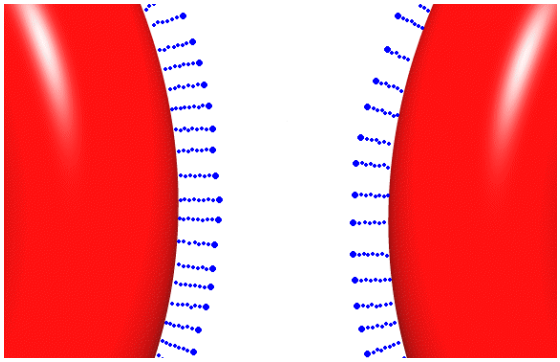
- **False positives** :
  - Presence of fibrin or bacterial contamination of the serum
  - Quality of the Coombs serum (adsorption of antibodies against human red blood cells).
  - Insufficient washed red blood cells or contaminated solution by quartz

1. **Haemolysis :**
   - Concentration of NaCl incorrect of physiological water
   - Centrifugation too fast,
   - …
2. **Rouleaux><agglutination :** check agglutination under a microscope (see microphotograpy page 8 for interpretation):
   - Chronical infections (rouleaux formation caused by plasmatic proteins increase).
   - Trypanosomiasis infection (presence of auto agglutinins and rouleaux formation).
   - **…**

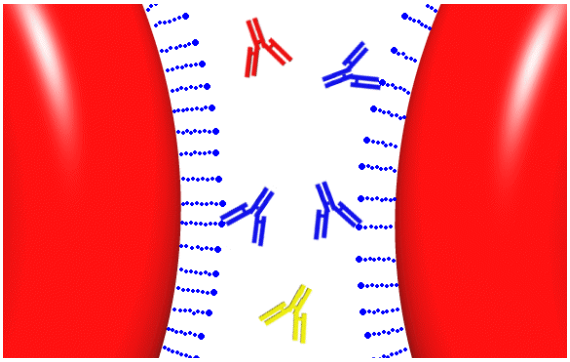
If a false positive can be excluded, it always concerns a dangerous antibody from the point of view of transfusion. **DO NOT TRANSFUSE, FIND ANOTHER DONOR**.

PRINCIPLE OF THE MAJOR COMPATIBILITY, INDIRECT COOMBS TEST

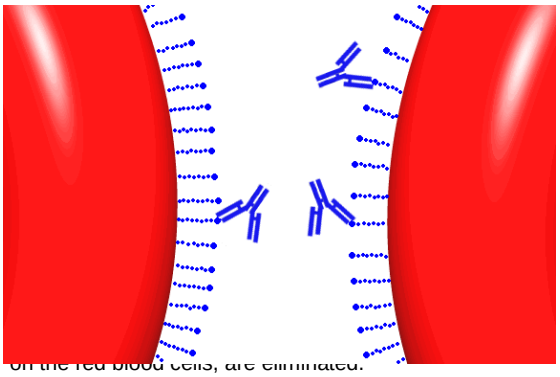




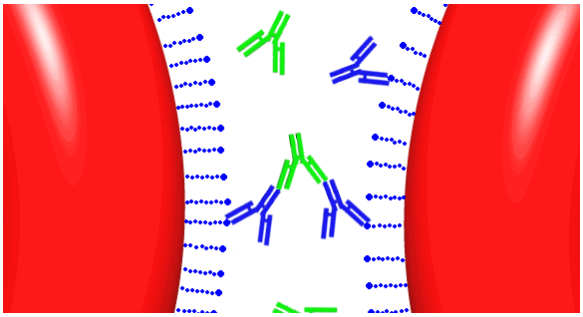
1. Antigens present on the surface of the red blood cells of the donor. (in blue for instance the antigens Fya).



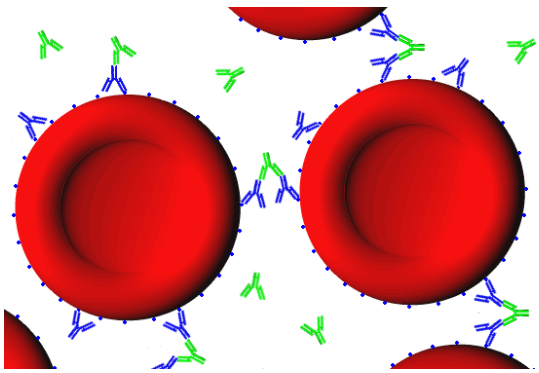
2. The serum of the receptor is added. The antibodies anti Fya, present in the serum will bind on the corresponding antigen. All the other present antibodies stay free in the serum**. Sensibilisation step.**



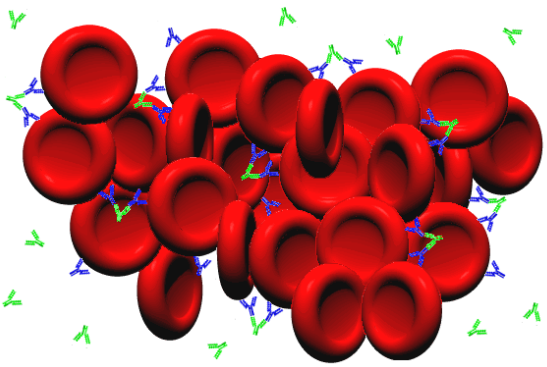
on the red blood cells, are eliminated.



4. After adding Coombs serum, the anti- human antibodies are binding with the anti-Fya antibody. **Revelation step.**



5. The fixation of the anti-human antibodies on two different anti-Fya antibodies are forming a “bridge” between the red blood cells resulting in an agglutination



6. This agglutination is macroscopically visible in the tube as a clot. Auto-test on the receptor

Auto test on the receptor
(In case of positive compatibility tests in saline medium)

The autotest can be executed in case the of a positive compatibility test in saline medium, in order to make the distinction between cold agglutinins and cold allo antibodies. As these two types of antibodies are not very dangerous in the context of transfusion and in practice one may transfuse the blood in both situations (**but at 37°C)**, this test is not very useful on district laboratory level.

Sample:

Serum of the receptor.
Red blood cells of receptor (blood taken on EDTA anticoagulant).

Reagents:

Physiological water [or saline solution] (cf. page 49).

Material:

Plastic haemolysis tubes of 10mm x 75mm, plastic Pasteur pipettes, bulb pipette for physiological water, haematological centrifuge, vacuum pump, fridge, microscope mirror [slides, cover slips 22mm x 22mm, microscope].

Technique:

1. Take a blood tube of the receptor (on EDTA).
2. Wash the red blood cells 3 times with physiological water.
3. Dilute the red blood cells at 5 % in physiological water.
4. Take 1 haemolysis tube.
5. Bring 2 drops of red blood cells of the receptor, washed 3 times and diluted to 5 %, in physiological water (point 3).
6. Add 2 drops of serum of the receptor.
7. Incubate 5 minutes at 22°C, centrifuge 1 minute at 1.000 RPM, read and evaluate the results.
8. Incubate 20 minutes at 4°C, centrifuge 1 minute at 1.000 RPM, read and evaluate the results.

A test is positive if there are agglutinations.

Table 4 Interpretations of an auto-test.

4°C	22°C	Interpretations
+	**+**	Presence of cold agglutinins (if major compatibility is also positive in saline medium).
+	**-**	Presence of cold agglutinins (if major compatibility is also positive in saline medium).
-	**-**	Presence of cold allo antibodies (if major compatibility is also positive in saline medium).
-	**+**	Incorrect test

+ = agglutination, - = absence of agglutination

Test of minor compatibility

Examination of antibodies of the donor versus red blood cells of the receptor. **This test makes only sense for transfusions of blood in iso-group** (presence of antibodies anti-A and anti-B in the serum of a person of group O).

This test is only rarely executed in routine in a small laboratory. Taken into account that the minor test detects the antibodies of the donor and that these are much diluted in the circulation of the receptor, this test is of restricted interest.

Of course, in case of plasma transfusion, the minor compatibility test is the most important.

The executing of the test is similar to the major compatibility, but by inversing donor and receptor. Washed red blood cells of the receptor are brought in contact with the serum of the donor.

TRANSFUSION AT A DISTRICT HOSPITAL: USEFUL TABLES

Table 5 : *Comparison of different (major) compatibility tests useful on district laboratory level.*

Type of compatibility test	Principle	Advantages	Inconveniences
« Rapid Cross match »	Mix on a slide a drop of **whole blood** of the donor and a drop of **whole blood** of the receptor. Observe eventual agglutination of the red blood cells.	▪ Detection of **ABO errors** (and irregular antibodies of IgM type). ▪ Fast (+/- 2 minutes). ▪ Easy. ▪ Feasible on the bed side of the patient. ▪ Neither equipment nor electricity needed. ▪ Low costs. ▪ …	▪ Only applicable for **iso-group** transfusions. ▪ Detects only IgM, so almost exclusively ABO errors. ▪ …
« Improved Cross match » [16]	Mix on a slide or in a tube, a drop of **whole blood** of the donor + a drop of **serum** of receptor. Observe eventual agglutination of red blood cells.	▪ Detection of **ABO errors** (and irregular antibodies of IgM type). ▪ Also applicable for transfusions of **non iso-groups**. ▪ Moderate fast (+/10 minutes). ▪ Moderate easy. ▪ …	▪ Detects only IgM, so almost exclusively ABO errors. ▪ Necessitates an **electric centrifuge.** ▪ Necessitates a big enough quantity of receptor blood (serum) → Small anaemic children? ▪ …
Major Compatibility (in saline medium)	Mix in a tube 2 drops of washed red blood cells of donor + 2 drops of serum of the receptor. Incubate 5 minutes at room T° (22°C), next centrifuge 1 minute at 1.000 rpm (100 *g*). Observe eventual agglutination of the red blood cells.	▪ Detection of **ABO errors** (and of irregular antibodies of IgM type). ▪ Also applicable for transfusions of **non iso-groups**. ▪ …	▪ Detects only IgM, so almost exclusively ABO errors. ▪ Necessitates a big enough quantity of receptor blood (serum) → Small anaemic children? ▪ Necessitates an **electric centrifuge.** ▪ Slow (+/30 minutes). ▪ Rather complex. ▪ …
Polybrene Method on slide[17] (cf. article in annex 4 page 64)	On a slide, 1 drop of washed and diluted at 20 % blood of the donor + 2 drops of serum of the receptor + 3 drops of a medium with weak ionic strength. Mix for 1 minute next add 1 drop of Polybrene, mix and observe eventual agglutination of red blood cells.	▪ Detection of **ABO errors** (and irregular antibodies of IgM type and of certain **IgG**). ▪ Also applicable for transfusions of **non iso-groups**. ▪ Low costs ▪ Rather fast (+/- 30 minutes). ▪ …	▪ Bad detection of certain irregular antibodies of IgG type (antibody anti-Kell for instance, so feasible in Asia, but not often in Africa or in Europe) ▪ Necessitates an **electric centrifuge.** ▪ Necessitates a big enough quantity of receptor blood (serum) → Small anaemic children? ▪ Reagents rather difficult to prepare locally. ▪ Rather complex. ▪ …
Major compatibility in saline medium + Coombs indirect in albumin medium)	Rather complex, see protocol compatibility in LISS- albumin medium page 49, but when using albumin instead of LISS-albumin, the incubation time has to be increased.	▪ Detection of **ABO errors** and irregular antibodies of IgM and **IgG** type. ▪ Also applicable for transfusions of **non iso-groups**. ▪ …	▪ Sensitivity and specificity not as good as in LISS-albumin medium. ▪ Necessitates a big enough quantity of receptor blood (serum) → Small anaemic children? ▪ Necessitates a **centrifuge** and a water bath (**electricity**). ▪ Moderate expensive. ▪ Rather complex. ▪ Slow (+/- 60 minutes). ▪ …
Major compatibility in saline medium + indirect Coombs in LISS-albumin medium)	Rather complex, see protocol page 49 .	▪ Detection of **ABO errors**, of irregular antibodies of IgM and **IgG** type. ▪ Also applicable for transfusions of **non iso-groups**. ▪ Rather fast (+/- 30 minutes). ▪ High sensitivity. ▪ High specificity. ▪ …	▪ Necessitates a big enough quantity of receptor blood (serum) → Small anaemic children? ▪ Necessitates a **centrifuge** and a water bath (**electricity**). ▪ Rather expensive ▪ Rather complex. ▪ …

[16] Une autre amélioration supplémentaire est d'utiliser des globules rouges lavés du donor, ceci complique et rallonge le test pour un résultat équivalent.
[17] Marie Lin. Compatibility testing without a centrifuge : the slide polybrene method. Transfusion 2004; 44: 410-413.

Table 6 : *Use of compatibility tests on district laboratory level described in the notes.*

Type of test	Use	Demonstrated antibodies	Questions / Actions	Necessary Equipment(s)		
				Electricity	Centrifuge	Water bath
Rapid Cross Match.	Transfusion of whole blood or of concentrated red blood cells: In Iso-group.	IgM in the receptor against the red blood cells of the donor : ABO Error Cold Auto antibodies Cold Allo antibodies	¿ Error of **ABO** grouping? **Do not transfuse if +**			
Major compatibility test in **saline** medium**.**	Transfusion of whole blood or of concentrated red blood cells: In iso-group. In non iso-group.	IgM in the receptor against the red blood cells of the donor : ABO Error Cold Auto antibodies Cold Allo antibodies	¿ Error of **ABO** grouping? ¿ Does the receptor have antibodies (**IgM**) against red blood cells of donor? If + and ABO error excluded**, transfuse at 37°C**	**X**	**X**	
M**ajor** compatibility test in **indirect Coombs** in **LISS-albumin** medium**.**	Transfusion of whole blood or of concentrated red blood cells: In iso-group. In non iso-group.	IgG in the receptor against the red blood cells of the donor : Warm Allo antibodies	¿ Does the receptor have antibodies (**IgG**) against red blood cells of donor? **Do not transfuse if +**	**X**	**X**	**X**
Minor compatibility test in **saline** medium**.**	Transfusion of whole blood or of concentrated red blood cells: In iso-group. Transfusion of plasma : In iso-group. In non iso-group.	IgM in the donor against the red blood cells of the receptor : ABO Error Cold Auto antibodies Cold Allo antibodies	¿ Does the receptor have antibodies (**IgM**) against red blood cells of donor? Whole blood or concentrated red blood cells : If + and ABO error excluded**, transfuse at 37°C** Plasma or concentrated red blood cells : **Do not transfuse if +**	**X**	**X**	
Minor compatibility test in **indirect Coombs** in **LISS-albumin** medium**.**	Transfusion of whole blood or of concentrated red blood cells: In iso-group. Transfusion of plasma : In iso-group. In non iso-group.	IgG in the donor against the red blood cells of the receptor : Warm Allo antibodies	¿ Does the receptor have antibodies (**IgG**) against red blood cells of donor? Whole blood or concentrated red blood cells : **Do not transfuse if +** Plasma : **Do not transfuse if +**	**X**	**X**	**X**

Table 7 : *Blood transfusion on district hospital level: from minimum until extra possibilities.*

Activities	The minimum	More… (in function of possibilities, listed for each activity in order of importance)
Blood donor	• None remunerated familial blood donors.	• Living blood bank. • Mini blood bank for emergencies (1 to 2 blood bags). • Blood bank based on none remunerated and regular voluntary blood donors. • …
Selection of donors	• Questionnaire and clinical selection.	• Delay of reflexion between the first screening test and the first blood donation (for the voluntary donors). • …
Type of serological screening	• Rapid tests **after/during** the taking of the blood unit.	• Rapid tests **before** the blood taking. • …
Serological screening on the donors	• HIV (antibodies). • HBsAg (antigens). • Syphilis (non treponemal test - RPR).	• HIV (antigens). • HCV (antibodies). • Syphilis (Test treponemal - TPPA type). <u>And in function of the region a screening for :</u> o Chagas Disease ? o African Trypanosomiasis ? o Leishmaniasis ? o Microfilaria ? • …
Type de transfusion	• « Warm » transfusion of whole blood o Iso-group (general rule). o Non iso-group (exception).	• «Cold» transfusion of whole blood (blood bank). • Transfusion of packed cells (blood bank). • [Transfusion of plasma (blood bank)]. • …
Blood grouping	• ABO grouping: Forward blood grouping • Rhesus grouping: Limited to antigen D on slide.	• ABO grouping: Reverse blood grouping. • ABO grouping in tube. • Rhesus grouping in tube. • …
Compatibility test	• Rapid Cross Match.	• Major compatibility test in saline medium associated with an indirect Coombs in LISS-albumin medium. • [Minor compatibility test]. • [Auto-tests]. • …

ANNEX 1

Wash of the red blood cells

The purpose of the wash of the red blood cells is to eliminate all the plasmatic antibodies which are free or non specifically fixed on the surface of the red blood cells.

Sample:

Blood taken on anticoagulant (EDTA).

Reagents:

Physiological water (or saline solution cf. preparation page 49).

Material:

Plastic haemolysis tubes 10mm x 75mm,
Plastic pipettes Pasteur,
Bulb pipette for physiological water,
Haematological centrifuge,
[Vacuum pump].

Technique :

1. Centrifuge sample 5 minutes at 3000 RPM.
2. Aspire the plasma with a vacuum pump or take off with a Pasteur pipette.
3. Bring 1 volume of the cell clot in the haemolysis tube.
4. Add at least 10 volumes of physiological water to the red blood cells clot with a bulb pipette (use the pressure of the pipette to bring the red blood cells in suspension).
5. Centrifuge the tube 5 minutes at 3000 RPM.
6. Aspire the plasma with a vacuum pump or take off with a Pasteur pipette.
7. Repeat two times steps 4 to 6.

ANNEX 2

Screening of dangerous O donors

It can happen, in case of emergency, that it is necessary to transfuse O blood to a receptor A, B or AB. The plasma of certain O persons may contain an important quantity of antibodies anti A or more rarely anti B which may cause a reaction with the red blood cells A, B or AB of the receptor. These persons, dangerous O donors, must be detected and may not be considered as “universal donors”. Blood of « dangerous O donors » may only be transfused to receptors of group O. The screening of dangerous O donors is only possible in the context of a blood bank and should be systematically performed at the moment of the arrival of the pocket of the O blood in the fridge (duration of the test). Nevertheless, for reason of rareness of dangerous O donors and the maximal use of iso-group transfusion, this test is little useful.

The fresh serum of an O donor is incubated with small quantities of red blood cells A_1 and B. If there are too many antibodies anti A or anti B these red blood cells will be haemolysed and the serum will be pink stained.

Sample:

Serum of the donor.
Known red blood cells A_1 and B, diluted at 5 % in physiological water.

Reagents:

Physiological water (or saline solution, cf. preparation page 49).

Material:

Plastic haemolysis tubes of 10mm x 75mm
plastic Pasteur pipettes
bulb pipette for physiological water
haematological centrifuge
vacuum pump
fridge
microscope mirror [slides, cover slips 22mm x 22mm, microscope].

Technique :

1. Take the serum of the donor O to be tested. The serum must be used within 6 hours.
2. Wash the known red blood cells A_1 and B 3 times with physiological water.
3. Dilute these red blood cells at 5 % in physiological water.
4. Take 2 haemolysis tubes.
5. Bring in a tube 1 drop of red blood cells A_1 diluted to 5 % and in the other 1 drop of red blood cells B diluted to 5 %.
6. Add to each tube 9 drops of serum of the donor O to be tested.
7. Incubate 2 hours at 37°C.
8. Bring the red blood cells back in suspension by slightly tapping the haemolysis tubes.
9. Centrifuge 1 minute at 1.000 RPM.
10. Control the colour of the supernatant.

If the serum is **yellow**, with sediment of red blood cells, this donor O can be considered as **universal**.

If the serum is **pink**, with limited sediment of red blood cells, this donor O must be considered as « dangerous donor ». His blood may only be transfused to a receptor of group O.

ANNEX 3

Screening for infectious diseases

Blood transfusion is known to be an efficient way for transmitting infectious diseases. It is therefore important to screen blood before its potential use in order to discard any blood unit capable of infecting a recipient.

Viral and related diseases :

- Hepatitis B, C, (A)? D,E G, (VHA), VHD, VHE, VHG/VGB-C
- HIV 1 / 2 (Human Immunodeficiency Virus).
- HTLV 1 / 2 (Human T-Lymphocytotrophic Virus).
- CMV (Cytomegalovirus).
- EBV (Epstein Barr virus).
- TTV (TT virus)
- HHV-6, HHV-8 (human herpes virus type 6 and 8)
- SEN-V (SEN virus
- HPB19 (human parvovirus)
- Creutzfeld-Jacob disease (and other prions).
- [Hemorrhagic fevers].
- …

Parasitical disease :

- Malaria.
- Leishmaniasis.
- Toxoplasmosis.
- Chagas disease.
- African trypanosomiasis.
- Babesiosis.
- (Microfilaria).
- …

Bacterial diseases :

- Syphilis
- Borreliosis.
- Brucellosis.
- …

Bacterial contamination of blood products. This is another often observed risk disorder directly associated with blood transfusion. Most commonly associated with contamination during blood collection or during handling of blood products, and on occasion, associated with bacterial infection of the donor, it is sometimes recognizable by obvious changes in the appearance of the blood product. When grossly contaminated, blood appears haemolysed and dark in colour.

Sometimes, infectious agents can be detected directly in blood (for example HBs antigen detection reflects directly an HBV infection). More often blood will be analyzed in order to detect specific antibodies. For some infectious diseases, the presence of antibodies may reflect a past infection and does not mean that the blood is infectious (hepatitis for example); in other cases, on contrary, antibodies may reflect a current transmissible infection (for example anti-HIV antibodies).

The latency, characterizing some infections, has also to be taken into account for two main reasons : first, the latent phase is often infectious, secondly, tests detecting viral antigens before antibodies become detectable are not always available (for example HCV).

In many developing countries, the prevalence of infectious diseases in the general population is high. For that reason, high rates of infected blood donors can be expected, and proportionally, a high rate of co-infections. Laboratory testing of blood donors for infectious diseases is therefore an essential phase in assessing blood safety. On the other hand, this high prevalence will also increase the risk of missing some detection because of the latency (windows period and/or sensitivity limitation)… This problem can be partially solved by a good donor's selection.

LEVELS OF SCREENING STRATEGY

Laboratory testing should be considered at least at three levels:

1. Screening tests applied to blood units: To be useful in the improvement of blood safety, screening tests have to be applied systematically on all blood units in order to identify any potentially dangerous blood. In this context, a positive test result is by itself a sufficient reason to discard the blood unit from therapeutic use. **Therefore, for the purpose of blood safety, the most sensitive test should be recommended for the screening of blood units.**

2. Laboratory testing may acquire a role of diagnosis when blood donors ask for the results obtained by analyzing their blood for infectious agents. In this context, the results of screening tests have to be confirmed by confirmatory methods with high specificity.

3. The results performed in a blood bank can also be used as indicators of effectiveness of the selection criteria applied to blood donors. Indeed, the rate of sample found to be "reactive" with the screening test will give information about the prevalence in the selected population and **may help to revise and / or reorient the criteria used in order to recruit and select "safer" blood donors.**

PARAMETERS INFLUENCING THE SCREENING STRATEGY

Before dealing with technical considerations, one should keep in mind that environmental parameters as well as some intrinsic characteristics of the infectious agents themselves are likely to have an effect on the prevention of transmissible diseases in developing countries.

- Ideally, any blood for transfusion purposes should be tested for the presence of all those agents which are prevalent in a given population, and if transmitted, can cause serious disease for the recipient.
- Epidemiological data (if available) in the local population have to be taken into account.
- In endemic areas, the probability for an adult recipient, being infected prior to transfusion, and to have achieved immunity, depends on the prevalence of the disease in the population…This is not true for young children…
- Some infectious agents are only present in cells and are not transmitted by cell free blood components such as plasma (malaria for example) [this aspect is to be considered only if separation of blood components is feasible]. Other agents are present and infectious in cells and cell free components.
- Some infectious agents are killed or at least their virulence is weakened after blood storage for 72 hours at 4 to 7 °C (syphilis, trypanosomes). This could be kept in mind if storage is feasible and safe.
- Information given to blood donors in order to teach them about at-risk behaviour and to encourage them to "self-deferral" is less expensive, less dangerous and probably as useful as testing to discard dangerous blood units (more relevant for sexually transmitted diseases).

When financial support is limited, local priority should be given to various screening tests according to the prevalence of the carrier state in the general population, the consequences of infection for the recipient's health and the age of recipient.

WHICH TESTS CAN BE USED IN A REMOTE AREA ?

TECHNICAL CONSIDERATION

- Number of blood transfusions ?

  Blood bank type ?
  Cold blood ?
  Test before /during / after blood unit's collection ?

- Test availability ?
- Test complexity ?
- Time ?
- Human resources ?
- …

HIV : In 2005 the WHO estimated that 5 % of HIV infections in Africa might be caused by transfusion. Therefore HIV screening is mandatory. A single positive screening test result is sufficient to decide to discard the blood unit. If the donor is to be informed, all precautions should be taken: the positive result should lead to performance of alternative tests according the adapted strategy of the prevalence. Sensitive, specific and rather cheep rapid tests are available (detection of antibodies and/or antibodies and antigens).

HBV : Hepatitis B is an important transfusion hazard since it is established that blood infected with hepatitis B virus is infectious in almost 100 % of cases. In developing countries, the rate of people infected with HBV is most of the time very high and may reach 90 % in adults. It is essential that every blood unit should be screened for HBsAg for the following reasons: a great number of transfusion indications refers to paediatric patients who have not been immunized; besides, the consequences of transfusing blood infected with hepatitis B, to immunized individuals are not known.

HCV : Few epidemiological data are available for developing countries. Screening for HCV antibodies is 2 times more expensive than for HIV. HCV is responsible for more than 90 % of post transfusion hepatitis, if HBV has been excluded (European data). Estimates are that 80 % of the persons receiving a transfusion with blood infected with HCV will seroconvert, and probably more than 50 % of the persons who seroconvert will develop chronic liver disease with possible serious complications 10 to 20 years after infection (liver cirrhosis, hepatic cellular carcinoma).

HBA : Hepatitis A has rarely been associated with transfusion, and the infection is clinically mild; screening whole blood donors is not anticipated.

CMV : The prevalence of the CMV antibody ranges from 50% to 80% of the population. Blood contaminated with CMV can cause problems in neonates or immune compromised patients. Potential problems in selected patient populations can be prevented by transfusing CMV negative blood. Donor blood is not routine tested for CMV. Tests are expensive and complex.

HTLV 1 / 2 : No systematic screening recommended except in areas where the disease is frequent (epidemiological data are incomplete, but there are three known high prevalence areas : Central and South America and the Caribbean, southern Japan and sub-Saharan Africa). Risk of transmission in the United States at this time is said to be 1 in 641,000. The risk of developing HTLV-1 disease, adult T-cell leukaemia/lymphoma or tropical spastic paraparesis, is estimated to 1 or 2 per 1.000 HTLV-1 positive cases per year after an incubation period averaging 20 years. The actual estimates are that about 60 % of the persons receiving blood containing HTLV-1 will seroconvert. The test (EIA and particle agglutination assay) gives many false positive results. Screening and confirmation tests are expensive and complex.

Malaria : The best method for the diagnosis of malaria is to examine a thick blood film for parasites. However, since this method requires microscopic examination of each sample, it is not suitable on a large scale. Even in endemic areas, the absence of parasites in a thick blood film, will not say that the blood is not infected (sensitivity limitation). Antibody detection is not applicable in endemic countries. In endemic areas, a medical history, seeking evidence of recent fever and illness is essential. The use of therapeutic (or prophylactic) anti-malarial drug for transfusion recipients has to be considered.

Chagas disease : As far as blood transmission of Chagas disease by blood is concerned, the problem is most serious in South America. However, migration of people from endemic to non endemic areas has resulted in the presence of infectious individuals in previously non endemic areas (America). No systematic screening is recommended except in areas where the disease is frequent. Laboratory testing in the early phase of infection is by examination of tick blood film in order to detect the protozoa. In the acute phase, the parasite can be cultured from blood samples. None of these two methods is applicable to the screening of blood donors. Several serological tests are available for the detection of antibodies that are produced in 50 % of acute phase patients, and in 95 % with chronic infection, but their sensitivity and specificity remain questionable. In some *T. cruzi* endemic areas, gentian violet is added to donor blood (125 mg / 500 ml blood) followed by storage at 2-8 °C for 24 hours to kill the parasite. Guidelines regarding the most appropriate test to use in a particular area should be obtained from the nearest Chagas disease reference laboratory.

African trypanosomiasis : African trypanosomiasis can be transmitted when donor blood contains *T.b. gambiense* (or less probably *T. b. rhodesiense*). It can occur in areas of high prevalence but very few instances have been reported (Quality of the data ?). The CATT test may be useful in endemic areas. If donor is to be informed, all precautions should be taken: the positive result should lead to perform of confirmation tests.

Leishmaniasis : Cases of transfusion-associated leishmaniasis are growing each year world wide. This is increasingly associated with patients who are positive for HIV. Transfusion-associated leishmaniasis requires that the parasites be present in the peripheral blood of the donor, survive processing and storage in the blood bank, and infect the recipient. In endemic areas for visceral leishmaniasis, where the population of potentially infected individuals may be much higher, a serological screening process should be used. These tests are expensive, they take a long time and are a little bit complex.

Microfilaria can be transmitted in blood and may cause allergic reactions but the larvae are unable to develop further in the recipient and therefore filariasis cannot occur. Wet blood examination may be used to detect infected blood.

Syphilis : Testing for syphilis (RPR or VDRL) is recommended. However, a positive result does not always mean that the blood unit is infectious. Besides, retention of blood for 3 days at 4°C inactivates the infecting agent. Although the risk of post-transfusion is quite low, the screening of infected donors may be used as a marker of individual risk of STD infections (HIV, …) on account of their sexual behaviour. If the donor is to be informed, all precautions should be taken : the positive result should lead to performance of a confirmation test.

Borreliosis : In areas with endemic recurrent fever, a good donor selection is the best manner to exclude the risk. The best method for diagnosis of recurrent fever is to examine a thick blood film for bacteria. However, since this method requires microscopic examination of each sample, it is not suitable for a large scale. Even in endemic areas, the absence of bacteria in a thick blood film doesn't mean that the blood is not infectious (sensitivity limitation).

…

Distribution of some infectious markers (example):

	RWANDA 1991 (n = 500)		CONGO 2005 (n= 2500)	
Marker	**POSitives**	**%**	**POSITIVES**	**%**
HIV	18	3,5	199	8
HBsAg	30	6	175	7
Syphilis	15	3,0	50	2
HCV Ab	15	3,0	(113) ?	4,5 (on 250) [18]
total	78		537	

Co-infections :

Rwanda 18. As much as 12 % of blood units collected must be discarded and not transfused.
Congo 134. As much as 16 % of blood units collected must be discarded and not transfused.

[18] N = 250

ANNEX 4

Compatibility test on slide, Polybrene method

Compatibility testing without a centrifuge: the slide Polybrene method

Marie Lin

BACKGROUND: A simple and rapid slide Polybrene method (SP) for pretransfusion compatibility testing is described. SP is particularly suitable for use in developing countries where, due to limited resources, centrifuges and biologic reagents may not be readily available.
STUDY DESIGN AND METHODS: The original manual Polybrene method (MP) was modified for use on glass microscope slides, eliminating the need for test tubes and centrifugation. The sensitivity of SP for detecting alloantibodies to RBC antigens was compared with that of MP and the IAT.
RESULTS: Both SP and MP were more sensitive than the IAT for detecting anti-E. SP detected 21 of 23 examples of anti^{v}-MI^{v} and 7 of 8 examples of anti-E. Kidd and Diego system antibodies were also readily detectable by SP, although the reactions were weaker than those observed with both MP and IAT. However, both SP and MP failed to detect some examples of antibodies to Kell system antigens.
CONCLUSIONS: SP is an acceptable method for compatibility testing in developing countries, particularly in populations where the frequency of K is low (e.g., southeast Asia). The reagents are inexpensive and can be prepared in-house. SP is simple to use, does not require a centrifuge, and can be performed by personnel with minimal training.

ABBREVIATIONS: MP = manual Polybrene; SP = slide Polybrene.

From the Immunohematology Reference Laboratory, Mackay Memorial Hospital, Taipei, Taiwan.
Address reprint requests to: Marie Lin, MD, FCAP, Immunohematology Reference Laboratory, Mackay Memorial Hospital, 92, Sec 2, Chuong Shan N Road, Taipei, Taiwan; e-mail: marilin@ms2.mmh.org.tw.
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Most developed countries have introduced standardized methods for pretransfusion testing that require the use of specific reagents and equipment (e.g., antiglobulin serum, centrifuges, etc.). However, for underdeveloped countries such items may be unavailable or too expensive to purchase. At a recent "Manual Polybrene" Workshop in Laos (December 2000, Lao Red Cross Blood Center, Vientiane)[1] and an "Immunohematology in Taiwan" Workshop in Vietnam (December 2002, Viet-Duc University Hospital, Hanoi), it was discovered that very few centrifuges were available for use in the transfusion services of these countries. Therefore, the development of a compatibility testing procedure that did not require the use of a centrifuge or expensive reagents was of particular importance.

Past experience in developing a national blood program for Taiwan in the 1960s[2] demonstrated the importance of having a simple, rapid, and inexpensive method for compatibility testing to standardize pretransfusion testing procedures. Before 1980, transfusion medicine was an area of low priority in Taiwan and only a few teaching hospital blood banks had adequate equipment, finance, and staff to incorporate the more expensive and time-consuming standard Western procedures. However, in 1983 the manual Polybrene (MP)[3] method, which is a simple and rapid procedure for the detection of RBC alloantibodies, was introduced at Mackay Memorial Hospital. Within a few years, MP was incorporated successfully into routine pretransfusion testing procedures throughout the whole of Taiwan (including both large and small institutions).[4]

In this report, the slide Polybrene (SP) method, which is even simpler to perform than MP, is described. The reagents required for the SP method are inexpensive and can be prepared in-house. In addition, blood-bank personnel require only minimal training to perform the test satisfactorily, and the use of a centrifuge is not required. SP also demonstrates good sensitivity for the detection of all antibodies that are of clinical significance in southeast Asia.

MATERIALS AND METHODS

Reagents

Low-ionic-strength medium was prepared as for the MP method described by Lalezari and Jiang.[3]

A 10-percent stock solution of Polybrene (hexadimethrine bromide, Sigma Chemical, St. Louis, MO) in normal saline was also prepared as described by Lalezari and Jiang.[3] A 0.1-percent working Polybrene solution was prepared by appropriate dilution of the 10-percent stock solution in saline.

Resuspending solution for reversing the nonspecific Polybrene induced aggregation was prepared by mixing 60 mL 0.4 mol/L trisodium citrate solution and 40 mL 5-percent dextrose.

(Note: the working Polybrene solution and trisodium citrate concentrations used in SP were double the concentrations that were used in MP.)

SP method

Sensitization phase. An ellipse, 3 × 1.5 cm in size, is drawn on a glass microscope slide with a wax pencil to prevent overflowing of reagents. Three drops of low-ionic-strength medium are added to the slide followed by 2 drops of test serum (or plasma) and 1 drop (50 µL) of 20 percent RBCs in saline (or 10 µL packed RBCs). The reagents are mixed thoroughly with an applicator stick and incubated at room temperature (~22°C) for 1 minute.

Polybrene aggregation phase. One drop of the 0.1-percent Polybrene working solution is then added to the reagents on the slide, mixed with an applicator stick, and incubated at room temperature for 1 minute. RBC aggregation usually begins to appear about 30 seconds after the addition of the 0.1-percent Polybrene solution and is complete within 1 minute.

Resuspension phase. One drop of resuspending solution is added, and the slide is gently rocked by hand for about 10 seconds until any nonspecific Polybrene-induced aggregation has dispersed. True antibody-induced agglutination does not dissociate and can be readily observed macroscopically. For weaker reactions, the agglutinates can be read using a magnifier. Results should be evaluated as soon as possible, and certainly no later than 3 minutes after the resuspending solution has been added.

Controls. Daily quality controls should include a weakly reacting anti-E or anti-D as a positive control and inert AB serum as a negative control.

MP method

MP was performed as described by Lalezari and Jiang[3] except that no supplementary antiglobulin phase was performed. Tests were examined microscopically.

IAT

A standard saline IAT was performed by incubating at 37°C for 30 minutes, followed by washing and the addition of antihuman IgG (Gamma Biologicals, Houston, TX). Tests were examined microscopically.

RESULTS

A comparison of the sensitivities of SP, MP, and IAT for the detection of various alloantibodies are shown in Tables 1 and 2.

From Table 1, it can be seen that SP and MP are more sensitive than IAT for the detection of anti-E. However, IAT is more sensitive than both SP and MP for the detection of anti^{v}-MI^{v}, -K, -Jk^{a}, and -JK^{b}.[5]

From Table 2, it can be seen that SP detected 21 of 23 examples of anti^{v}-MI^{v} and 7 of 8 examples of anti-E. More significantly, SP readily detected all ABO incompatibilities (Table 2). SP also readily detected other important alloantibodies of clinical significance, including antibodies against antigens of the Kidd and Diego blood group systems, although reactions were weaker than those observed with MP and IAT.

TABLE 1. Comparison of alloantibody titers by SP, MP, and IAT

Methods*	Anti-E	Anti-E	Anti-E	Anti-E	Anti-E	Anti-E	Anti-E†	Anti-D†	Anti-MI^{a}	Anti-MI^{a}	Anti-MI^{a}	Anti-MI^{a}	Anti-MI^{a}
SP	neat	4	8	16	128	512	200	1600	neat	2	2	4	8
MP	neat	8	128	64	1024	2048	1600	6400	2	2	4	16	8
IAT	neat	8	2	neat	512	256	200	3200	neat	4	16	64	32

Methods	Anti-K‡	Anti-K‡	Anti-Jk^{a}‡	Anti-Jk^{a}‡	Anti-Jk^{a}‡	Anti-Di^{a}‡	Anti-Fy^{a}‡	Anti-Fy^{a}‡	Anti-Fy^{b}	Anti-Fy^{a}‡	Anti-Fy^{a}‡	Anti-Fy^{b}
SP	2	16	2	4	16	2	4	4	neat	4	4	neat
MP	neat	16	neat	4	16	16	64	16	16	64	16	16
IAT	64	512	4	8	32	4	128	8	4	128	8	4

* Diluents for antibody titration were 3 percent albumin (BSA, Sigma Chemical) in saline. Except for the two MoAbs initially diluted 1:100 and followed by 1:2 step dilutions, the rest of the antibodies were titrated in 1:2 dilution steps.
† FDA-approved commercial MoAbs.
‡ FDA-approved commercial antisera.

TABLE 2. Numbers of patients in whom antibodies were detected and methods of detection

Anti-	Number of patients	SP	MP	IAT
MI^{a}	23	21	23	23
E	8	7	8	7
D	5	5	5	5
C	1	1	1	1
Jk^{a}, Jk^{b}, Jk3	8	5	8	8
Kp^{b}	2	0	0	2
Di^{a}, Di^{b}	6	3	6	6
S	1	1	1	1
M	4	4	4	2
P1	2	2	2	0
Le^{a}, Le^{b}	2	2	2	1
A	10	10	10	10
B	10	10	10	10
H*	1	1	1	1
I	2	2	2	0
Total	85	74	83	77

* Bombay phenotype.

Among 85 antibodies from patients that were tested, 74 antibodies were detectable by SP, including 10 anti-A and 10 anti-B. Anti-Kp^{b} could only be detected by IAT (Table 2) and higher anti-K titers were obtained by IAT than by both SP and MP (Table 1). Therefore, the main disadvantage of the two Polybrene methods is that a small number of antibodies of the Kell blood group system will not be detected. However, because the frequency of K in oriental populations is very low, this is not clinically significant.

DISCUSSION

Slide methods have generally been considered inferior to tube methods with regards the detection of clinically significant alloantibodies. This is mainly due to the fact that in tube methods, RBCs are forced close together by centrifugal force, which thus enhances hemagglutination. However, in the SP method RBCs are brought close together by the action of the positively charged 0.1-percent Polybrene reagent, resulting in nonspecific RBC aggregation. Heparin interferes with the test, and 2 to 3 times of Polybrene should be added if heparinized samples are used (i.e., 2 or 3 drops of Polybrene). The Polybrene-induced aggregation can be quickly reversed by adding 1 drop of 0.4 mol/L citrate resuspending solution leaving any specific antibody-induced agglutination intact.

In this study, the sensitivity and efficacy of SP in detecting alloantibodies were compared simultaneously with MP and IAT by testing antibodies that were encountered during antibody screening and cross-matching in the Blood Bank, Mackay Memorial Hospital; antibodies obtained from the "Serum, Cells and Rare Fluids International Immunohematology Exchange Group" (SCARF), which included antibodies rarely found in Taiwan; two highly diluted commercial MoAb (anti-D and anti-E), which were routinely used as daily positive controls for SP and MP; and also several commerical antisera as shown in Table 1. The results show that SP detected most alloantibodies of clinical significance, especially anti-E and anti^{v}-MI^{v}. These two antibodies are the most common alloantibodies of clinical significance in Taiwan[6] and most likely also in the rest of southeast Asia. Anti-'MI^{v}' is used in Taiwan to describe antibodies that react with RBCs of the MiIII phenotype.[6] Other alloantibodies such as anti-D, -K, -Jk^{a}, -Jk^{b}, -Fy^{a}, and -Fy^{b} were also detected by SP with a sensitivity similar to that of MP. Although SP (and MP) are not as sensitive as IAT for the detection of anti-K, because most patients and donors in southeast Asia are K negative,[7,8] then the incidence of anti-K would be expected to be very rare. This is indeed the case, and during the past 20 years in Taiwan, only one antibody against an antigen of the Kell blood group system has been found (anti-Ku in a K$_{null}$ person).[9] Therefore, the implementation of a sensitive procedure for the detection of antibodies to antigens of the Kell blood group system would appear to be of low priority in routine compatibility procedures for southeast Asia.

SP is extremely rapid (about 5 min), cost effective (reagents for the test can be prepared simply and in-house), and is easy to perform. Personnel require only 1 day's training to perform the test with confidence. Therefore, the introduction of SP in countries with limited resources, and especially in countries where pretransfusion testing is limited to ABO grouping, will help significantly to improve transfusion safety. In such countries, many antibodies of clinical significance, which until now have been undetected, can now be detected.

Transfusion services in developing countries, not only lack centrifuges but also lack finances for purchasing the reagents and training new staff. In such situations, SP is the method of choice for routine pretransfusion testing so as to improve patient safety.

ACKNOWLEDGMENTS

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ANNEX 5

Indicative price list (Diamed, blood grouping) *http://www.diamed.ch/*

Items	Pkg size	Reference (Diamed)	Price (€) 09/2005	Number of tests[19]
Coombs-serum, polyvalent anti-IgG (rabbit), anti-C3d (monoclonal),Diaclon, green	10 ml	107140	29,0	100
LISS modified for red cell suspension Dialiss (Liss-albumin)	10 ml	106510	11,2	50
Anti-A or anti-B, blood grouping monoclonal IgM Diaclon for slide and tube test	10 ml	100810	8,5	200
Anti-AB, blood grouping monoclonal IgM Diaclon for slide and tube test	10 ml	100910	8,5	200
Anti-D, blood grouping monoclonal IgG and IgM antibodies, D(VI-) Diaclon for slide and tube test	10 ml	101070	17,7	200

Items	Package	Price (€) 08/2006	Number of feasible tests
Hematological centrifuge for blood bank : Immufuge II (Baxter©)	1	675	S.O

Indicative price list (serological screening):

Items	Package	Price (€) 03/2006	Number of feasible tests [20]
Determine HIV 1 / 2	100	80	100
Determine HBsAg	100	90	100
HCV SPOT	100	325	100
CATT	250	150	250
RPR card antigen suspension Becton Dickinson	500	150	500

[19] Without taking into account the controls and the losses.
[20] Without taking into account the controls and the losses.

ANNEX 6

HIV rapid test example

Determine™

HIV-1/2

This package insert must be read carefully prior to use. Package insert instructions must be followed accordingly. Reliability of assay results cannot be guaranteed if there are deviations from the instructions in this package insert.

NAME AND INTENDED USE

The Abbott Determine™ HIV-1/2 is an *In Vitro*, visually read, qualitative immunoassay for the detection of antibodies to HIV-1 and HIV-2 in human serum, plasma or whole blood. The test is intended as an aid to detect antibodies to HIV-1/HIV-2 from infected individuals.

SUMMARY AND EXPLANATION OF THE TEST

AIDS (Acquired Immunodeficiency Syndrome) is characterized by changes in the population of T-cell lymphocytes. In an infected individual, the virus causes depletion of helper T-cells, which leaves the person susceptible to opportunistic infections and some malignancies. The virus that causes AIDS exists as two related types known as HIV-1 and HIV-2. The presence of the AIDS virus elicits the production of specific antibodies to either HIV-1 or HIV-2.[1,2,3]

BIOLOGICAL PRINCIPLES OF THE PROCEDURE

Determine HIV-1/2 is an immunochromatographic test for the qualitative detection of antibodies to HIV-1 and HIV-2.

Sample is added to the sample pad. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid-antigen conjugate. This mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site.

If antibodies to HIV-1 and/or HIV-2 are present in the sample, the antibodies bind to the antigen-selenium colloid and to the antigen at the patient window, forming a red line at the patient window site.

If antibodies to HIV-1 and/or HIV-2 are absent, the antigen-selenium colloid flows past the patient window, and no red line is formed at the patient window site.

To insure assay validity, a procedural control bar is incorporated in the assay device.

CONTENTS

Abbott Determine HIV-1/2 Serum/Plasma Assay (List No. 7D23-12), 20 Tests

- Determine HIV-1/2 Test Card, 2 cards (10 tests/card), HIV-1/2 recombinant antigen and synthetic peptide coated.

Abbott Determine HIV-1/2 Whole Blood Assay (List No. 7D23-32), 20 Tests

- Determine HIV-1/2 Test Card, 2 cards (10 tests/card), HIV-1/2 recombinant antigen and synthetic peptide coated.
- 1 Bottle (2.5 mL) Chase Buffer (List No. 7D22-11) prepared in phosphate buffer. Preservatives: Antimicrobial Agents.

ACCESSORIES (required but not provided)

Serum/Plasma or Whole Blood (venipuncture assay)		Whole Blood (fingerstick assay)	
Pipette	No. 7D22-51	Lancets*	No. 7D22-31
Pipette Tips	No. 7D22-61	EDTA Capillary Tubes	No. 7D22-21

*Not available in European Union countries.

WARNINGS AND PRECAUTIONS

For *In Vitro* Diagnostic Use.

CAUTION:

Appropriate biosafety practices[4,5] should be used when handling specimens and reagents. These precautions include, but are not limited to the following:

- Wear gloves.
- Do not pipette by mouth.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in areas where these materials are handled.
- Clean and disinfect all spills of specimens or reagents using a suitable disinfectant, such as 0.5% sodium hypochlorite.[6,7]
- Decontaminate and dispose of all specimens, reagents, and other potentially contaminated materials in accordance with local regulations.[8,9]

STORAGE

The Abbott Determine HIV-1/2 Test Cards and Chase Buffer must be stored at 2-30°C until expiration date.

Kit components are stable until expiration date when handled and stored as directed. Do not use kit components beyond expiration date.

SPECIMEN COLLECTION

Serum, Plasma, and Whole Blood Collection by Venipuncture

Human serum, plasma, and whole blood collected by venipuncture should be collected aseptically in such a way as to avoid hemolysis.

NOTE: For whole blood and plasma specimens, EDTA collection tubes must be used.

Whole Blood Collection by Fingerstick[10]

Before collecting a fingerstick specimen, place an EDTA capillary tube on a clean dry surface.

1. Choose the fingertip of the middle, ring, or index finger (whichever is the least callused) for adults and children older than one year. Warm the hand as needed with a warm, moist towel or warm water to increase blood flow.

2. Clean fingertip with alcohol; allow to air dry. Position the hand palm-side up.

3. Use a new lancet for each person. Place the lancet off-center on the fingertip. Firmly press the lancet against the finger and puncture the skin. Dispose of the lancet in an appropriate biohazard sharps container.

4. Wipe away the first drop of blood with a sterile gauze pad.
5. Hold the finger lower than the elbow and apply gentle, intermittent pressure to the base of the punctured finger several times. Touch the tip of the EDTA Capillary Tube to the drop of blood*. Avoid air bubbles.

*If EDTA Capillary Tubes (No. 7D22-21) will be used, fill the tube with blood between the 2 marked lines.

SPECIMEN STORAGE

- Serum and plasma specimens should be stored at 2-8°C if the test is to be run within 7 days of collection. If testing is delayed more than 7 days, the specimen should be frozen (-20°C or colder).
- Whole blood collected by venipuncture should be stored at 2-8°C if the test is to be run within 7 days of collection. Do not freeze whole blood specimens.
- Whole blood collected by fingerstick should be tested immediately.

TEST PROCEDURE

The desired number of test units from the 10-test card can be removed by bending and tearing at the perforation.

NOTE: Removal of the test units should start from the right side of the test card to preserve the lot number which appears on the left side of the test card.

1. Remove the protective foil cover from each test.
2. For serum or plasma samples:
   a. Apply 50µL of sample (precision pipette) to the sample pad (marked by the arrow symbol).
   b. Wait a minimum of 15 minutes (up to 60 minutes) and read result.
3. For whole blood (venipuncture) samples:
   a. Apply 50 µL of sample (precision pipette) to the sample pad (marked by the arrow symbol).
   b. Wait one minute, then apply one drop of Chase Buffer to the sample pad.
   c. Wait a minimum of 15 minutes (up to 60 minutes) and read result.
4. For whole blood (fingerstick) samples:
   a. Apply 50 µL of sample (by EDTA capillary tube) to the sample pad (marked by the arrow symbol).
   b. Wait until blood is absorbed into the sample pad, then apply one drop of Chase Buffer to the sample pad.
   c. Wait a minimum of 15 minutes (up to 60 minutes) and read result.

QUALITY CONTROL

To insure assay validity, a procedural control is incorporated in the device and is labeled "Control". If the control bar does not turn red by assay completion, the test result is invalid and the sample should be retested.

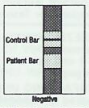
INTERPRETATION OF RESULTS

POSITIVE (Two Bars)

Red bars appear in both the control window (labeled "Control") and the patient window (labeled "Patient") of the strip. Any visible red color in the patient window should be interpreted as positive.

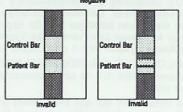
NEGATIVE (One Bar)

One red bar appears in the control window of the strip (labeled "Control"), and no red bar appears in the patient window of the strip (labeled "Patient").



INVALID (No Bar)

If there is no red bar in the control window of the strip, and even if a red bar appears in the patient window of the strip, the result is invalid and should be repeated. If the problem persists, contact your local Abbott Customer Service and Support Center.



NOTES:

- The test result is positive even if the patient bar appears lighter or darker than the control bar.
- If an invalid test result occurs repeatedly, or for technical assistance, contact your local Abbott Customer Service and Support Center.

LIMITATIONS OF THE PROCEDURE

- The Abbott Determine HIV - 1/2 test is designed to detect antibodies to HIV - 1 and HIV - 2 in human serum, plasma, and whole blood. Other body fluids or pooled specimens may not give accurate results.
- The intensity of the patient bar does not necessarily correlate to the titer of antibody in the specimen.
- A negative result with Determine HIV does not exclude the possibility of infection with HIV. A false negative result can occur in the following circumstances:
  - low levels of antibody (e.g., early seroconversion specimens) are below the detection limit of the test
  - infection with a variant of the virus that is less detectable by the Determine HIV assay configuration
  - HIV antibodies in the patient that do not react with specific antigens utilized in the assay configuration
  - specimen handling conditions which result in loss of HIV antibody multivalency

For these reasons care should be taken in interpreting negative results. Other clinical data (e.g., symptoms or risk factors) should be used in conjunction with the test results.

- Positive specimens should be retested using another method and the results should be evaluated in light of the overall clinical evaluation before a diagnosis is made.
- Whole blood or plasma specimens containing anticoagulants other than EDTA may give incorrect results.

PERFORMANCE CHARACTERISTICS

SPECIFICITY

A total of 1,594 serum and plasma specimens from Asia, West Africa, and North America were tested by Abbott Determine HIV-1/2 and a commercially available test (Table I).

Table I
Specificity of Abbott Determine HIV-1/2

Population	Number of Specimens Tested	Negative by Abbott Determine HIV-1/2	Negative by a Commercially Available Test
Seronegative Serum	908	907/908 (99.89%)	908/908 (100.00%)
Plasma	403	403/403 (100.00%)	403/403 (100.00%)
Pregnant Females	58*	57/57 (100.00%)	57/57 (100.00%)
West Africans	49	48/49 (97.96%)	48/49 (97.96%)
Disease States Other than HIV and Potentially Interfering Substances	176*	173/175 (98.86%)	174/175 (99.45%)
Total	1,594	1,588/1,592 (99.75%)	1,590/1,592 (99.87%)

*One specimen from a pregnant female and an HCV positive patient were positive by both Abbott Determine and the commercially available test. Both specimens confirmed positive by HIV-1 Western Blot.

A total of 368 seronegative whole blood specimens from Thailand were tested with paired serum and plasma by Abbott Determine HIV-1/2. Thirty-nine of the whole blood specimens were collected by both venipuncture and fingerstick (Table II).

Table II
A Comparison of Abbott Determine HIV-1/2 Specificity in Seronegative Whole Blood and Paired Serum and Plasma Specimens

Specimen Type	Number of Specimens Tested	Negative by Abbott Determine HIV-1/2
Serum	368	368/368 (100.00%)
Plasma	368	368/368 (100.00%)
Whole Blood (venipuncture)	368	368/368 (100.00%)
Whole Blood (fingerstick)	39	39/39 (100.00%)

SENSITIVITY

A total of 869 HIV-1 and HIV-2 antibody positive serum and plasma specimens from Asia, Africa, North and South America were tested by Abbott Determine HIV-1/2 and a commercially available test (Table III).

Table III
Sensitivity of Abbott Determine HIV-1/2

Population	Number of Specimens Tested	Positive by Abbott Determine HIV-1/2	Positive by a Commercially Available Test
HIV-1 Positive	521	521/521 (100.00%)	521/521 (100.00%)
HIV-2 Positive	114	114/114 (100.00%)	114/114 (100.00%)
HIV-1 Subtypes A-G	222	222/222 (100.00%)	Not Tested Not Tested
HIV-1 Group O	12	12/12 (100.00%)	Not Tested Not Tested
Total	869	869/869 (100.00%)	635/635 (100.00%)

A total of 102 seropositive whole blood specimens from Thailand were tested with paired serum and plasma by Abbott Determine HIV-1/2. Thirty-two of the whole blood specimens were collected by both venipuncture and fingerstick (Table IV).

Table IV
A Comparison of Abbott Determine HIV-1/2 Sensitivity in Seropositive Whole Blood and Paired Serum and Plasma Specimens

Specimen Type	Number of Specimens Tested	Positive by Abbott Determine HIV-1/2
Serum	102	102/102 (100.00%)
Plasma	102	102/102 (100.00%)
Whole Blood (venipuncture)	102	102/102 (100.00%)
Whole Blood (fingerstick)	32	32/32 (100.00%)

BIBLIOGRAPHY (See Back Page)

ANNEX 7

HBsAg rapid test example

Determine™
AgHBs

Lire attentivement cette notice avant l'utilisation du dosage. Les instructions d'utilisation doivent être suivies en conséquence. La fiabilité des résultats du dosage ne peut pas être garantie si ces instructions ne sont pas strictement respectées.

DEFINITION ET DOMAINE D'APPLICATION
Abbott Determine AgHBs est un dosage immunologique qualitatif *in vitro* à lecture visuelle pour la détection de l'antigène de surface de l'hépatite B (AgHBs) dans le sérum, le plasma ou le sang total humain. Ce test constitue une aide pour la détection de l'AgHBs chez les sujets infectés.

RESUME ET EXPLICATION DU TEST
Les dosages de l'AgHBs sont utilisés pour le dépistage de l'AgHBs dans le sang et les produits dérivés, afin d'éviter la transmission du virus de l'hépatite B (VHB) aux receveurs de ces produits. Les dosages de l'AgHBs sont également utilisés de façon routinière pour le diagnostic d'une infection par le VHB suspectée et le suivi de l'état des patients infectés, à savoir si l'infection du patient a été guérie ou si le patient est devenu un porteur chronique du virus.[1]

PRINCIPES BIOLOGIQUES DE LA METHODE
Determine AgHBs est un test immunochromatographique pour la détection qualitative de l'antigène de surface de l'hépatite B (AgHBs).

L'échantillon est déposé sur la zone de dépôt de l'échantillon. Comme l'échantillon migre jusqu'à la zone de dépôt du conjugué, il se reconstitue et se mélange avec le conjugué colloïde de sélénium-anticorps. Ce mélange continue à migrer sur la phase solide jusqu'aux anticorps immobilisés au niveau de la fenêtre-patient.

Si l'AgHBs est présent dans l'échantillon, l'antigène se lie à l'anticorps du conjugué anticorps-colloïde de sélénium et à l'anticorps de la fenêtre-patient en formant une ligne rouge.

Si l'AgHBs est absent, le conjugué anticorps-colloïde de sélénium traverse la fenêtre-patient sans former de ligne rouge.

Une barre de contrôle de la procédure est incluse dans ce système de dosage afin d'assurer la validité du test.

COMPOSITION
Dosage Abbott Determine AgHBs Sérum/Plasma (Réf. 7D25-13), 100 tests

- Test Determine AgHBs, 10 cartons (10 tests par carton) recouverts d'anticorps anti-HBs (souris, monoclonaux).

Dosage Abbott Determine AgHBs Sang total (Réf. 7D25-33), 100 tests

- Test Determine AgHBs, 10 cartons (10 tests par carton) recouverts d'anticorps anti-HBs (souris, monoclonaux).
- 1 flacon (2,5 ml) de tampon de fixation (Réf. 7D22-11) préparé dans du tampon phosphate. Conservateurs : Agents antimicrobiens.

ACCESSOIRES (nécessaires mais non fournis)

Sérum/Plasma ou Sang total (ponction veineuse)		Sang total (bout du doigt)	
Pipette	Réf. 7D22-51	Lancettes*	Réf. 7D22-31
Embouts pour pipette	Réf. 7D22-61	Tubes capillaires avec de l'EDTA	Réf. 7D22-21

* Non disponibles dans les pays de l'Union Européenne

PRECAUTIONS ET RESTRICTIONS D'EMPLOI
Pour diagnostic *in vitro*.
ATTENTION :
Les échantillons et réactifs doivent être manipulés conformément aux règles biologiques en vigueur.[2,3] Ces précautions comprennent, entre autres, les mesures suivantes :

- **Porter des gants.**
- **Ne pas effectuer de pipetages à la bouche.**
- **Ne pas manger, boire, fumer, ni manipuler des produits cosmétiques ou des lentilles de contact dans les locaux où sont manipulés ces matériaux.**
- **Nettoyer et désinfecter toutes les éclaboussures d'échantillons et de réactifs à l'aide d'un désinfectant antimicrobien tel qu'une solution d'hypochlorite de sodium à 0,5%.[4,5]**
- **Décontaminer et éliminer tous les échantillons, réactifs et autres substances susceptibles d'avoir été contaminées conformément à la réglementation en vigueur.[6,7]**

CONSERVATION
Les tests Abbott Determine AgHBs et le tampon de fixation doivent être conservés entre 2 et 30°C jusqu'à la date de péremption.

Les composants du kit sont stables jusqu'à la date de péremption s'ils sont conservés et manipulés selon les indications du fabricant. Ne pas utiliser les composants du kit au-delà de la date de péremption.

PRELEVEMENT DES ECHANTILLONS
Prélèvement de sérum, plasma et sang total par ponction veineuse
Le sérum, le plasma et le sang total humains prélevés par ponction veineuse doivent être recueillis dans des conditions d'asepsie, de manière à éviter l'hémolyse.

REMARQUE : Pour les échantillons de sang total, il faut utiliser des tubes de prélèvement avec de l'EDTA.
Prélèvement de sang total sur le bout du doigt[8]
Avant de prélever un échantillon sur le bout du doigt, placer un tube capillaire avec de l'EDTA sur une surface propre et sèche.

1. Pour les adultes et les enfants de plus d'un an, choisir le bout du majeur, de l'annulaire ou de l'index (choisir le moins calleux). Chauffer la main avec une serviette chaude et humide ou bien avec de l'eau chaude afin d'augmenter le flux sanguin.
2. Nettoyer le bout du doigt avec de l'alcool ; laisser sécher à l'air. Placer la main paume vers le haut.

3. Utiliser une lancette différente pour chaque personne. Placer la lancette sur un côté du bout du doigt. Appliquer une ferme pression sur la lancette placée sur le doigt et piquer la peau. Jeter la lancette dans un récipient pour déchets biologiques pointus.

4. Essuyer la première goutte de sang avec une gaze stérile.

5. Maintenir le doigt un peu plus bas que le coude et appliquer par intermittence de faibles pressions à la base du doigt piqué. Effleurer la goutte de sang avec l'extrémité du tube capillaire contenant de l'EDTA*. Eviter la formation de bulles d'air.

*Si l'on utilise les tubes capillaires contenant de l'EDTA (Réf. 7D22-21), remplir le tube de sang jusqu'à un niveau situé entre les 2 traits.

CONSERVATION DES ECHANTILLONS

- Si le test est effectué dans les 7 jours qui suivent le prélèvement, les échantillons de sérum et de plasma doivent être conservés entre 2 et 8°C. S'ils sont analysés plus de 7 jours après le prélèvement, ils doivent être congelés (à une température inférieure ou égale à -20°C).
- Si le test est effectué dans les 7 jours qui suivent le prélèvement, le sang total prélevé par ponction veineuse doit être conservé entre 2 et 8°C. Ne pas congeler les échantillons de sang total.
- Le sang total prélevé sur le bout du doigt doit être analysé immédiatement.

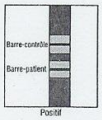
PROCEDURE D'ANALYSE
Le nombre souhaité de tests peut être détaché du carton de 10 tests en pliant et déchirant au niveau de la perforation.

REMARQUE : Détacher les tests en commençant par la droite du carton de tests afin de préserver le numéro de lo apparaissant sur la gauche de ce carton.

1. Enlever la protection plastique de chaque test.
2. Pour les échantillons de sérum ou de plasma :
   a. Distribuer 50 µl d'échantillon (à l'aide d'une pipette de précision) sur la zone de dépôt de l'échantillon (symbole : flèc
   b. Attendre au moins 15 minutes (maximum : 24 heures) et lire le résultat.
3. Pour les échantillons de sang total (ponction veineuse) :
   a. Distribuer 50 µl d'échantillon (à l'aide d'une pipette de précision) sur la zone de dépôt de l'échantillon (symbole : flèche).
   b. Attendre une minute, puis distribuer une goutte de tampon de fixation sur la zone de dépôt de l'échantillon.
   c. Attendre au moins 15 minutes (maximum : 24 heures) et lire le résultat.
4. Pour les échantillons de sang total (bout du doigt) :
   a. Distribuer 50 µl d'échantillon (avec un tube capillaire contenant de l'EDTA) sur la zone de dépôt de l'échantillon (symbole : flèche).
   b. Attendre que le sang soit absorbé par la zone de dépôt, puis distribuer une goutte de tampon de fixation sur la zon dépôt de l'échantillon.
   c. Attendre au moins 15 minutes (maximum : 24 heures) et lire le résultat.

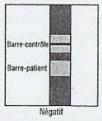
CONTROLE DE QUALITE
Un contrôle de la procédure annoté "Control" est inclus dans ce système afin d'assurer la validité du test. Si la barre de con ne vire pas au rouge à la fin du dosage, le résultat du test n'est pas valide et l'échantillon doit être réanalysé.

INTERPRETATION DES RESULTATS
POSITIF (deux barres)
Les barres rouges apparaissent dans la fenêtre-contrôle (annotée "Control") et la fenêtre-patient (annotée "Patient") sur la bandelette. Toute couleur rouge visible dans la fenêtre-patient doit être interprétée comme un résultat positif.



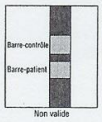
Positif

NEGATIF (une barre)
Une barre rouge apparaît dans la fenêtre-contrôle (annotée "Control"), la barre rouge de la fenêtre-patient (annotée "Patient") n'apparaissant pas sur la bandelette.

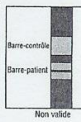


Négatif

NON VALIDE (pas de barre)
Si la barre rouge n'apparaît pas dans la fenêtre-contrôle de la bandelette et même si une barre rouge apparaît dans la fenêtre-patient de la bandelette, le résultat n'est pas valide et le test doit être recommencé. Si le problème persiste, contacter votre Service Clients Abbott.



Non valide



Non valide

REMARQUES :

- Le résultat du test est positif même si la barre-patient est plus claire ou plus foncée que la barre-contrôle.
- Si un résultat non valide venait à se répéter ou en cas de questions, contacter votre Service Clients Abbott.

LIMITES DE LA METHODE

- Le test Abbott Determine AgHBs est destiné à détecter l'antigène de surface de l'hépatite B (AgHBs) dans le sérum, plasma et le sang total humains. D'autres fluides physiologiques ou pools d'échantillons peuvent donner des résultat imprécis.
- L'intensité de la barre-patient n'est pas nécessairement proportionnelle au titre d'antigène de l'échantillon.
- Aucun test ne peut garantir de façon absolue qu'un échantillon ne contient pas de faibles concentrations en AgHBs, com celles présentes à un stade très précoce de l'infection. C'est pourquoi un résultat négatif n'exclut pas la possibilité d'u exposition à l'AgHBs ou d'une infection par celui-ci.
- Les échantillons de sang total contenant des anticoagulants autres que l'EDTA peuvent donner des résultats incorrect
- Afin de pouvoir diagnostiquer et distinguer une infection aiguë par le VHB d'une infection chronique, la détection de l'AgHBs doit être associée aux symptômes que présente le patient et aux autres marqueurs sérologiques viraux de l'hépa B.

CARACTERISTIQUES SPECIFIQUES
SPECIFICITE
Un total de 1908 échantillons de sérum, de plasma et de sang total provenant d'Asie, d'Afrique de l'Ouest et d'Amérique du N ont été analysés par les dosages Abbott Determine AgHBs et Abbott Dainascreen AgHBs (tableau I).

Tableau I
Spécificité du test Abbott Determine AgHBs

Population	Nombre d'échantillons analysés	Négatifs par Abbott Determine AgHBs		Négatifs par Abbott Dainascreen AgHBs	
Séronégatifs					
Sérum	682	681/682	(99,85%)	681/682	(99,85%)
Plasma*	498	498/498	(100,00%)	498/498	(100,00%)
Sang total	458	458/458	(100,00%)	458/458	(100,00%)
Femmes enceintes	58**	57/57	(100,00%)	57/57	(100,00%)
Africains de l'Ouest	50	50/50	(100,00%)	50/50	(100,00%)
Maladies autres que l'infection par le VHB et substances potentiellement interférentes	162**	159/159	(100,00%)	159/159	(100,00%)
Total	1908	1903/1904	(99,95%)	1903/1904	(99,95%)

*Pour 92% (458/498) des échantillons pour lesquels le plasma et le sang total étaient disponibles, les résultats étaient concorda

**Un échantillon provenant d'une femme enceinte et 3 échantillons positifs pour le VIH-1 étaient positifs par les tests Determ et Dainascreen. Ces 4 échantillons ont été confirmés positifs par le dosage Abbott AxSYM AgHBs.

SENSIBILITE
Un total de 434 échantillons de sérum et de sang total séropositifs pour l'AgHBs, provenant d'Asie et d'Amérique du Nord ont analysés par les dosages Abbott Determine AgHBs et Abbott Dainascreen AgHBs (tableau II).

Tableau II
Sensibilité du test Abbott Determine AgHBs

Population	Nombre d'échantillons analysés	Positifs par Abbott Determine AgHBs		Positifs par Abbott Dainascreen AgHBs	
Positif pour l'AgHBs					
Sérum	373	353/373	(94,64%)	336/373	(90,08%)
Sang Total	61	60/61	(98,36%)	N/A	N/A
Total	434	413/434	(95,16%)	336/373	(90,08%)

BIBLIOGRAPHIE (Se référer à la dernière page)

ANNEX 8

HCV rapid test example

ONE STEP Anti-HCV Test

SD BIO LINE HCV

1. Explanation of the test

Hepatitis C virus (HCV) now is recognized as a major agent of chronic hepatitis, transfusion-acquired non-A, non-B hepatitis and liver disease throughout the world. HCV is an enveloped positive-sense, single-stranded RNA virus. Clinical diagnostic issues related to HCV is the detection of HCV antibodies in human serum, plasma or whole blood by immunoassay. We have constructed HCV genes for the expression of recombinant antigens in bacterium systems such as *E. coli* and focused on structural and non-structural regions of HCV-encoded polyprotein, which are definitely immunogenic. The major immunoreactive antigens of these proteins have been reported as core, NS3, NS4 and NS5 regions of HCV genome, which are known to be highly immunodominant regions. For diagnosis of HCV infection, these recombinant proteins were used as capture materials of a immunochromatographic (rapid) test. Compared to the first generation HCV test using single recombinant antigens, multiple antigens using recombinant proteins have been added in new serologic tests to avoid non-specific cross-activity and to increase the sensitivity of the HCV antibody test.
The SD BIOLINE HCV test is a immunochromatographic (rapid) test for the qualitative detection of antibodies specific to HCV, in human serum, plasma or whole blood.
The SD BIOLINE HCV test contains a membrane strip, which is pre-coated with recombinant HCV capture antigen (core, NS3, NS4 and NS5) on test band region. The protein A - colloid gold conjugate and serum sample moves along the membrane chromatographically to the test region (T) and forms a visible line as the antigen-antibody-protein A gold particle complex forms with high degree of sensitivity and specificity. This test device has a letter of T and C as "Test Line" and "Control Line" on the surface of the case. Both the Test Line and Control Line in result window are not visible before applying any samples. The Control Line is used for procedural control. Control line should always appear if the test procedure is performed properly and the test reagents of control line are working.

2. Materials provided

1) SD BIOLINE HCV test device
2) Assay Diluent
3) Instructions for use

3. Precautions

The SD BIOLINE HCV test devices should be stored at room temperature. The test device is sensitive to humidity and as well as to heat. Perform the test immediately after removing the test device from the foil pouch. Do not use it beyond the expiration.

4. Specimen collection and storage

1) [whole blood] Collect the whole blood using the suitable anti-coagulant.
2) [serum or plasma] Centrifuge whole blood to get plasma or serum specimen.
3) If specimens are not immediately tested they should be refrigerated at 2~8 degrees C. For storage periods greater than three days, freezing is recommended. They should be brought to room temperature prior to use.
4) Specimens containing precipitate may yield inconsistent test results. Such specimens must be clarified prior to assaying
5) The whole blood may be used for testing immediately or may be stored at 2~8 degrees C up to three days.

5. Warnings

1) For in vitro diagnostic use only.
2) Do not eat or smoke while handling specimens.
3) Wear protective gloves while handling specimens. Wash hands thoroughly afterwards.
4) Avoid splashing or aerosol formation.
5) Clean up spills thoroughly using an appropriate disinfectant.
6) Decontaminate and dispose of all specimens, reaction kits and potentially contaminated materials, as if they were infectious waste, in a biohazard container.
7) Do not use the test kit if the pouch is damaged or the seal is broken.

6. Procedure of the test

1) Remove the test device from the foil pouch, and place it on a flat, dry surface.
2) Add 10 μL of serum, plasma or whole blood to the sample well, and then add 3 drops of assay diluent (Figure 1).
3) As the test begins to work, you will see purple color move across the results window in the center of the test device.
4) Interpret test results at 5~20 minutes. Do not interpret test result after 20 minutes.

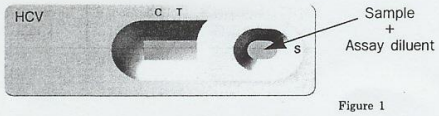


Figure 1

Caution : The above interpreting time is based on reading the test results at room temperature of 15 to 30 degrees C. If your room temperature is significantly lower than 10 degrees C, then the interpreting time should be properly increased.

7. Interpretation of the test

1) A color band will appear in the left section of the result window to show that the test is working properly. This band is the Control Band.
2) The right section of the result window indicates the test results. If another color band appears in the right section of the result window, this band is the Test Band.

Negative results : The presence of only one band within the result window indicates a negative result (Figure 2).

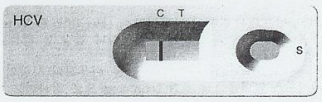


Figure 2

Positive results : The presence of two color bands ("T" band and "C" band) within the result window, no matter which band appears first, indicates a positive result (Figure 3).

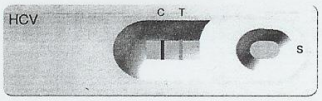


Figure 3

Invalid results : If the purple color band is not visible within the result window after performing the test, the result is considered invalid (Figure 4). Some causes of invalid results are: not following the directions correctly or the test may have deteriorated beyond the expiration date. It is recommended that the specimen be re-tested using a new test kit.

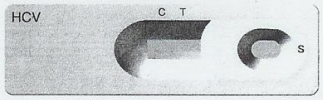


Figure 4

Note : A positive result will not change once it has been established at 20 minutes. However, in order to prevent any incorrect results, the test result should not be interpreted after 20 minutes.

8. Limitations of the test

A negative result does not preclude the possibility of infection with HCV. Other clinically available tests are required if questionable results are obtained. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.

9. Performance Characteristics

1) Sensitivity and Specificity
The SD BIOLINE HCV have tested with positive and negative clinical samples tested by a leading commercial anti-HCV ELISA test. The result shows that the SD BIOLINE HCV is very accurate to other commercial ELISA kit.

Reference		SD BIOLINE HCV		Total Results
Method	Result	Positive	Negative	
Commercial ELISA	Positive	206	2	208
	Negative	3	497	500
Total Results		209	499	708

In a comparison of the SD BIOLINE HCV versus a leading commercial anti-HCV ELISA test, results gave sensitivity of 99.0% (206/208), a specificity of 99.4% (497/500), and a total agreement of 99.3% (703/708).
2) Precision
(1) Within run precision was determined by using 10 replicates of four different specimens containing different concentrations of antibody. The negative and positive values were correctly identified 100% of the time.
(2) Between run precision was determined by using the four different specimens containing different concentrations of antibody in 3 different replicates with 3 different lots of test devices. Again negative and positive results were observed 100% of the time.

10. Bibliography of suggested reading

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SD STANDARD DIAGNOSTICS, INC.
575-34 Pajang-dong, Jangan-ku, Suwon-si, Kyonggi-do, Korea

ANNEX 9

RPR test example

BD Macro-Vue. RPR Card Tests ver: 26/09/2005

18 mm Circle Qualitative and Quantitative

Brewer Diagnostic Kit for the Serologic Detection of Syphilis

INTENDED USE

The ***Macro-Vue.*** RPR (Rapid Plasma Reagin) 18 mm Circle Card Test is a nontreponemal testing procedure for the serologic detection of syphilis.1,2

SUMMARY AND EXPLANATION

The ***Macro-Vue*** RPR Teardrop Card Test (using finger puncture blood) was the original Card Test and was developed for field use where testing could be performed without laboratory equipment.3,4 By incorporating machine rotation, ringed test surfaces, and certain other technical changes, the RPR Circle Card Test was developed for use in large scale testing in public health and clinical laboratories. The RPR 18 mm Circle Card Test is recommended when venous blood collection is employed and a large volume of serum is available, such as generally prevails in public health and clinical laboratories.5-12 When a specimen contains antibody, flocculation occurs with a coagglutination of the carbon particles of the RPR Card antigen, which appear as black clumps against the white background of the plastic-coated card. By contrast, nonreactive specimens appear to have an even light-gray color. In special situations when nontreponemal test results are needed rapidly and the specimen is collected as EDTA plasma, the RPR 18 mm Circle Card Test can be used if the test is performed within 24 h.13,14

PRINCIPLES OF THE PROCEDURE

RPR Card antigen suspension is a carbon particle cardiolipin antigen1 which detects “reagin", an antibody-like substance present in serum or plasma from syphilitic persons, and occasionally in serum or plasma of persons with other acute or chronic conditions. The reagin binds to the test antigen, which consists of cardiolipin-lecithin-coated cholesterol particles, causing macroscopic flocculation.

REAGENT

The ingredients* of the RPR Card antigen suspension are1: 0.003% cardiolipin, 0.020-0.022% lecithin, 0.09% cholesterol, 0.0125 M EDTA, 0.01 M Na2HPO4, 0.01 M KH2PO4, 0.1% thimerosal (preservative), 0.02% charcoal (specially prepared, BD), 10% choline chloride, w/v, and deionized/distilled water. *Adjusted and/or supplemented as required to meet performance criteria.

Warnings and Precautions:

For *in vitro* Diagnostic Use. Pathogenic microorganisms, including hepatitus viruses and Human Immunodeficiency Virus, may be present in clinical specimens. Standard Precautions.15-18 and institutional guidelines should be followed in handling all items contaminated with blood and other body fluids.

Antigen: Refrigeration is recommended for the RPR Card antigen suspension only. Storage in bright sunlight or temperatures above 30°C should be avoided; such conditions may cause a rough appearance of the antigen when used with nonreactive sera. If the ampule of antigen is frozen during shipment, it can be reconstituted once by warming to room temperature; avoid repeated freezing and thawing. Immediate use of a refrigerated antigen may result in decreased sensitivity of the test. Therefore, upon
removal from the refrigerator, allow the antigen to warm to room temperature (23 to 29°C) before use. Do not use antigen beyond the expiration date.

Diagnostic Test Cards: Specially prepared, plastic-coated cards designed for use with the RPR Card antigen. In handling, take care not to fingermark the card test areas, as this may result in an oily deposit and improper test results. When spreading specimen within confines of test areas, avoid scratching the card with the ***Dispenstirs “*** device or stirrer” If the specimen does not spread to the outer perimeter of test area, use another test area of card.

Dispenstirs. and Capillaries: In performing the Card Tests, a ***Dispenstirs*** device (18 mm Circle qualitative test only) or capillary may be used to transfer the specimen to the card surface. A new ***Dispenstirs*** device or capillary must be used for each test specimen. When transferring from the collecting tube, the specimen must not be drawn up into the rubber bulb attached to the capillary, as this will cause incorrect readings on subsequent tests.

Needles: To maintain clear passage for accurate drop delivery, upon completion of the tests, remove the needle from the dispensing bottle and rinse the needle with deionized/distilled water. Do not wipe the needle since this will remove the silicone coating and may affect the accuracy of the drop of antigen being dispensed.

Reading of Card Test Results: Read immediately following rotation in the “wet” state under a high intensity incandescent lamp or strong daylight.

Rotation: The recommended speed for mechanical rotation is 100 ± 2 rpm. The rotator should circumscribe a circle approximately two centimeters in diameter in the horizontal plane. A moistened humidifying cover should be used to prevent drying of test specimens during rotation.

Storage of Antigen: Refrigerate at 2 to 8°C. All other components of the kit should be stored in a dry place at room temperature in the original packaging. See “Warnings and Precautions” for additional information. Once placed in the *dispensing bottle* (provided in each kit) and refrigerated (2 to 8°C), the antigen reactivity remains satisfactory for approximately three months, or until the expiration date, if it occurs sooner. Label the dispensing bottle with the antigen lot number, expiration date, and date antigen was placed in the bottle.

SPECIMEN COLLECTION AND PREPARATION

No special preparation of the patient is required prior to specimen collection.

To Test Unheated Serum: Collect blood by venipuncture into a clean, dry tube without anticoagulant and allow to clot. Centrifuge the specimen at a force sufficient to sediment cellular elements. Keep the serum in the original collecting tube or transfer the serum into a clean, dry test tube if testing is to be delayed. Serum, removed from the clot, may be refrigerated at 2 to 8°C, for up to 5 days or frozen at -20°C or below in a Pyrex (or equivalent) vial or capped test tube.1 Avoid repeated freeze.thawing of specimens.

To Test Heated Serum: After collection and centrifugation, as for unheated serum, transfer to a clean dry tube and place in 56°C water bath, or a heat block for 30 min.
To Test Unheated Plasma: Collect blood by venipuncture into a tube containing anticoagulant such as EDTA, heparin, potassium oxalate, potassium sequestrene or sodium fluoride. EDTA and heparin have the advantage of not being critical with respect to concentration; as little as 1 mL of blood in a tube normally used to collect 7 mL of blood produces satisfactory results. With the other anticoagulants, it is advisable to collect no less than one half a tube of blood. Centrifuge as above. Keep plasma in the original collecting tube, and if stored, store the specimen at 2 to 8°C. Test specimen within 24 h of blood collection.

PROCEDURES AND RESULTS
Materials Provided: Various RPR Card Test kits are available (see .Availability.) which contain sufficient card antigen suspension to perform the specified number of daily control card and card tests, and the required dispensing bottle, dispensing needle, cards and either capillaries, stirrers, or ***Dispenstirs*** devices.
Materials Required But Not Provided:
1. Controls with established patterns of graded reactivity should be included in each day's testing to confirm optimal reactivity of the antigen. See "Availability" for ***Macro-Vue*** RPR 18 mm Circle Card Test Control Cards.
2. A rotator, 100 ± 2 rpm, circumscribing a circle 2 cm in diameter, with automatic timer, friction drive, and a cover containing a moistened sponge or blotter.
3. Saline (0.9%) for use in quantitative testing. Prepare by adding 900 mg dry sodium chloride, ACS to 100 mL deionized/distilled water.
4. Serum Nonreactive to syphilis in 0.9% saline; required for diluting test specimens giving a Reactive result at the 1:16 dilution. Also required is the necessary equipment and labware used in preparation, storage and handling of serologic specimens.

Preliminary Preparations: Review "Warnings and Precautions" and "Specimen Collection and Preparation" prior to performance of card tests. When tests are to be performed, the antigen suspension should be checked with controls of graded reactivity using the particular test procedure. Only those antigens which give the prescribed reactions should be used. Controls, RPR Card antigen suspension and test specimens should be at room temperature when used. Before use, vigorously shake the ampule for 10 to 15 s to resuspend the antigen and disperse any carbon particles lodged in the neck of the ampule. If any carbon should remain in the neck of the ampule after this shaking, no additional effort should be made to dislodge it as this will only tend to produce a coarse antigen. Check delivery of the needle by placing the needle firmly on a 1 mL pipet or syringe; fill the pipet or syringe with antigen suspension, and holding the pipet or syringe in a vertical position, count the number of drops delivered in 0.5 mL. The correct number of drops is given in the table opposite: Attach the needle to the tapered fitting on the dispensing bottle. Be sure the antigen is below the breakline; snap the ampule neck and withdraw all of the antigen into the dispensing bottle by collapsing the bottle and using it as a suction device. Label the dispensing bottle with the antigen lot number, expiration date, and date antigen was placed in the bottle. Shake the antigen dispensing bottle gently before each series of antigen droppings. *The needle and dispensing bottle should be discarded when the kit is used up. It is imperative techniques as described herein be followed in detail.*

Test Method	Color of Needle Hub	Number of Drops in 0.5 mL
18 mm Circle	Yellow, 20 G	30 ± 1 drop

18 mm Qualitative Card Test Using Dispenstirs. Devices :
1. Hold a ***Dispenstirs*** device between thumb and forefinger near the stirring or sealed end. Squeeze and do not release pressure until open end is below surface of specimen, holding the specimen tube vertically to minimize stirring up of cellular elements when using original blood tube. Release finger pressure to draw up the sample.
2. Holding in a vertical position directly over the card test area to which the specimen is to be delivered (not touching card surface), squeeze ***Dispenstirs*** device allowing one drop to fall onto card (approx. 0.05 mL; *each* ***Dispenstirs*** *device is designed to expel slightly in excess of 0.05 mL to compensate for small amount of specimen retained by stirring end*).
3. Invert ***Dispenstirs*** device and with sealed stirring end, spread the specimen filling entire surface of circle. (If desired, sample remaining may be discharged into specimen tube from which it was drawn.) Discard ***Dispenstirs*** device. Repeat procedure for number of specimens to be tested.
4. Gently shake antigen dispensing bottle before use. Holding in a vertical position, dispense several drops in dispensing bottle cap to make sure the needle passage is clear. Place one "free-falling" drop (20 G, yellow hub needle) onto each test area. *Do not restir; mixing of antigen and specimen is accomplished during rotation*. Pick up the pre-dropped antigen from bottle cap.
5. Rotate for 8 min (± 30 s) under humidifying cover, on mechanical rotator at 100 ± 2 rpm. Following rotation, to help differentiate Nonreactive from Minimally Reactive results, a brief rotating and tilting of the card by hand (3 or 4 to-and-fro motions) must be made. Immediately read macroscopically in the "wet" state under a high intensity incandescent lamp or strong daylight. Report as: Reactive : Showing characteristic clumping ranging from slight but definite (minimal-to-moderate) to marked and intense. Nonreactive : Showing no clumping. See the Reading Guide.
Note: *There are only two possible final reports with the Card Test : "Reactive or Nonreactive" regardless of the degree of reactivity. Reactivity minimal-to-moderate (showing slight, but definite clumping) is always reported as Reactive. Slightly granular or "rough" reactions should be repeated using an alternative procedure. For donor screening, these tests may bereported as "indeterminant" pending further evaluation. See "Limitations of the Procedure".* All reactive syphilis tests should be repeated using an alternative procedure.

18 mm Qualitative Card Test Using Capillaries:
1. Using a new capillary, attach rubber bulb to capillary and remove 0.05 mL of specimen from blood collecting tube by allowing specimen to rise to measuring line on capillary, taking care not to transfer cellular elements. (If desired, a serologic pipette may be used, but do not pipette by mouth.)
2. Place measured specimen onto circle of diagnostic test card, by compressing rubber bulb, while holding one finger over the hole in the bulb.
3. Using a new stirrer (broad end) for each specimen, spread to fill entire circle. Discard stirrer. Repeat procedure for number of specimens to be tested.
4. Gently shake antigen dispensing bottle before use. Holding in vertical position, dispense several drops in dispensing bottle cap to make sure the needle passage is clear. Place one "free-falling" drop (20 G, yellow hub needle) onto each test area. *Do not restir; mixing of antigen and specimen is accomplished during rotation*. Pick up the pre-dropped antigen from bottle cap.
5. Rotate for 8 min (± 30 s) under humidifying cover, on mechanical rotator at 100 ± 2 rpm. Following rotation, to help differentiate Nonreactive from Minimally Reactive results, a brief rotation and tilting of the card by hand (3 or 4 to-and-fro motions) must be made. Immediately read macroscopically in the "wet" state under a high intensity

incandescent lamp or strong daylight. Report as: Reactive : Showing characteristic clumping ranging from slight but definite (minimum-to-moderate) to marked and intense. Nonreactive : Showing no clumping. See the Reading Guide.
Note: There are only two possible final reports with the Card Test : Reactive or Nonreactive, regardless of the degree of reactivity.Reactive minimal-to-moderate (showing slight, but definite clumping) is always reported as Reactive. Slightly granular or "rough" reactions should be repeated using an alternative procedure. For donor screening, these tests may be reported as "indeterminant" pending further evaluation. See Limitations of the Procedure. All reactive syphilis tests should be repeated using an alternative procedure.

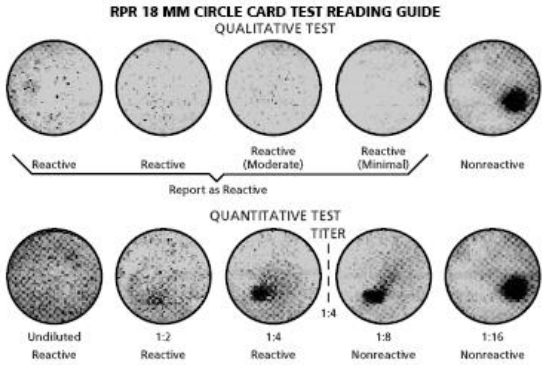
18 mm Circle Quantitative Card Test:
1. For each specimen to be tested, place 0.05 mL of 0.9% saline onto circles, numbered 2 to 5. A capillary (red line), or serological pipette, 1 mL or less, may be used. DO NOT SPREAD SALINE!
2. Using a capillary (red line graduated at 0.05 mL, to the tip) with rubber bulb attached, place 0.05 mL of specimen onto circle 1.
3. Refill capillary to red line with test specimen, and holding in a vertical position, prepare serial two-fold dilutions by drawing saline and test specimen mixture up-and-down capillary 5 to 6 times. Avoid formation of bubbles. Transfer 0.05 mL from circle 2, to 3, to 4, to 5, mixing after each transfer. Discard 0.05 mL after mixing contents in circle 5.
4. Using a new stirrer (broad end) for each specimen, start at highest dilution of serum (circle 5) and spread serum, filling the entire surface of circle. Proceed to circles 4, 3, 2 and 1 and accomplish similar spreading.
5. Gently shake antigen dispensing bottle before use. Holding in vertical position, dispense several drops in dispensing bottle cap to make sure needle passage is clear. Place one "free-falling" drop (20 G, yellow hub needle) onto each test area. *Do not restir; mixing of antigen and specimen is accomplished during rotation*. Pick up the pre-dropped antigen from bottle cap.
6. Rotate for 8 min (± 30 s) under humidifying cover, on mechanical rotator at 100 ± 2 rpm. Following rotation, to help differentiate Nonreactive from Reactive minimal-to- moderate (RM) results, a brief rotating and tilting of the card by hand (3 or 4 to-and-fro motions) must be made. Immediately read macroscopically in the wet state under a high intensity incandescent lamp or strong daylight. Report in terms of the highest dilution giving a Reactive including minimal-to-moderate reaction.

Examples:
(Prozone reaction : see "Limitations of the Procedure")

R = Reactive
N = Nonreactive
RM = Reactive minimal-to-moderate

(Und.) 1:1	1:2	1:4	1:8	1:16	Report
RM	N	N	N	N	Reactive, 1:1 dilution
R	R	R	N	N	Reactive, 1:4 dilution
R	R	R	R	N	Reactive, 1:8 dilution

Unheated or Heated Serum: If the highest tested (1:16) is Reactive, proceed as follows.
1. Prepare a 1:50 dilution of Nonreactive serum in 0.9% saline. (This is to be used for making 1:32 and higher dilutions of specimens to be quantitated)
2. Prepare a 1:16 dilution of the test specimen by adding 0.1 mL of serum to 1.5 mL of 0.9% saline. Mix thoroughly.
3. Place 0.05 mL of 1:50 Nonreactive serum in circles 2, 3, 4, and 5.
4. Using capillary, place 0.05 mL of 1:16 dilution of test specimen in circle 1.
5. Refill capillary to red line, make serial two-fold dilutions and complete tests as described under steps 3 to 6. (See "18 mm Circle Quantitative Card Test") Higher dilutions are prepared if necessary in 1:50 Nonreactive serum.
Plasma: If a baseline is to be established from which changes in titer can be determined, the test should be repeated on unheated serum (see section "Unheated Serum").
Reading and Reporting the Macro-Vue. RPR Card Tests: Individual reactions should be evaluated in the "wet" state, under a high intensity incandescent lamp or strong daylight. Immediately following rotation read and record as Reactive or Nonreactive.



Quality Control
Quality control requirements must be performed in accordance with applicable local, state and/or federal regulations or accreditation requirements and your laboratory's standard Quality Control procedures. It is recommended that the user refer to pertinent NCCLS guidance and CLIA regulations for appropriate Quality Control practices.

LIMITATIONS OF THE PROCEDURE
The diagnosis of syphilis should not be made on a single reactive result without the support of a positive history or clinical evidence. Therefore, as with any serological testing procedure, Reactive card test specimens should be subjected to further serologic study. Serum specimens which are Reactive in qualitative testing should be quantitated to establish a baseline from which changes in titer can be determined, particularly for evaluating treatment.1 The use of plasma specimens to establish a baseline from which changes in titer can be determined has not been evaluated. False-negative results can occur because of failure to recognize prozone reactions. Prozone reactions occur in 1% to 2% of patients with secondary syphilis. These specimens may exhibit a nonreactive pattern that is slightly granular or .rough. Upon dilution, the reactivity will increase and then decrease as the endpoint titer is approached. All tests with a rough appearance should be further evaluated. False-

negative nontreponemal test results are also seen in incubating primary and late syphilis.1 It is not necessary to perfom the quantitative procedure on reactive donor samples. The RPR Card Tests cannot be used for testing spinal fluids. The ideal specimen for neonatal testing is the infant's serum as obtained by heel stick procedure. However, cord blood may be used for baseline screening when no other specimen is available.1 With cardiolipin type antigens, biological false positive reactions have been reported in diseases such as infectious mononucleosis, leprosy, malaria, lupus erythematosus, vaccines and virus pneumonia. In leprosy, Portnoy3 reported no false positives; Achimastos19 reported 14 of 50 leprosy cases were Reactive and Scotti20 reported 1 out of 208 cases was reactive with RPR Card which were nonreactive with the FTA-ABS and TPI tests. Dorwart 21 studied the incidence of chronic BFP reactions in various connective tissue disorders. Six out of 41 cases of systemic lupus erythematosus were reactive in the Card Test, whereas only 5 were reactive in the VDRL slide test. Only 1 out of 23 cases of rheumatoid arthritis was reactive with both RPR Card and VDRL slide tests. In pregnancy, several reports indicated the occurrence of false positive reactions.11, 22 Narcotic addiction and autoimmune diseases also may give false positive reactions.23 Pinta, yaws, bejel and other treponemal diseases produce positive reactions in this test.1 Lipemia will not interfere with the card tests, however, if the degree of lipemia is so severe as to obscure the state of the antigen particles, the specimen should be considered unsatisfactory for testing. Do not test specimens that are grossly hemolyzed, contaminated or extremely turbid; report as "Specimen unsatisfactory for testing".1

EXPECTED VALUES AND PERFORMANCE CHARACTERISTICS

RPR Card antigen suspension is tested for the established pattern of reactivity against reference antigen suspensions and meets the U.S. Centers for Disease Control and Prevention (CDC) product specifications for performing the RPR 18 mm Circle Card Tests. These performance characteristics were established from a large number of papers which have appeared in the scientific literature, from routine daily test performances in syphilis serology testing laboratories and are in conformity with CDC specifications.

Reported studies show the RPR Card Tests have adequate sensitivity and specificity in relation to clinical diagnosis and a reactivity level similar to that of the VDRL slide test.6,10, 24, 25 Heating of serum specimens at 56°C for 30 min has been shown to have no effect on reactivity.20 A qualitative comparison of 1104 simultaneously collected serum and EDTA plasma specimens was conducted using the ***Macro-Vue*** RPR 18 mm Circle Card Test. There was complete agreement in test results which included 134 reactive and 970 nonreactive pairs. In other studies comparable results were found between plasma and serum pairs (306 specimens) with RPR Card Tests both in qualitative and quantitative procedures.14, 26

AVAILABILITY

Cat. No. Description

Macro-Vue. RPR Card Tests:

274449	Kit No. 104: (300 qualitative tests), contains: two 3 mL amps. antigen, 20 G needle, dispensing bottle, 350 stirrers, 30 cards with ten 18 mm Circle spots ea. and 300-0.05 mL capillaries.
275005	Kit No. 110: (500 qualitative tests), contains: three 3 mL amps. antigen, 20 G needle, dispensing bottle, 50 cards with ten 18 mm Circle spots ea. and 500-0.05 mL ***Dispenstirs.*** devices.
275239	Kit No. 112: (150 quantitative tests), contains: five 3 mL amps. antigen, 20 G needle, dispensing bottle, 200 stirrers, 50 cards with fifteen 18 mm Circle spots ea. and 150-0.05 mL capillaries.
275539	Kit No. 115: (150 qualitative tests), contains: one 3 mL amp. antigen, 20 G needle, dispensing bottle, 15 cards with ten 18 mm Circle spots ea. and 150-0.05 mL ***Dispenstirs.*** devices.
275110	Bulk Kit No. 510: (5,000 qualitative tests).
275692	Bulk Kit No. 532: (10,000 qualitative tests).
276709	***Macro-Vue.*** RPR Card Test Control Cards containing graded reactivity specimens, (R, RM and N 18 mm circles). Box of 10.
272905	***Dispenstirs.*** (single use, plastic pipettes), 0.05 mL, Box of 500.

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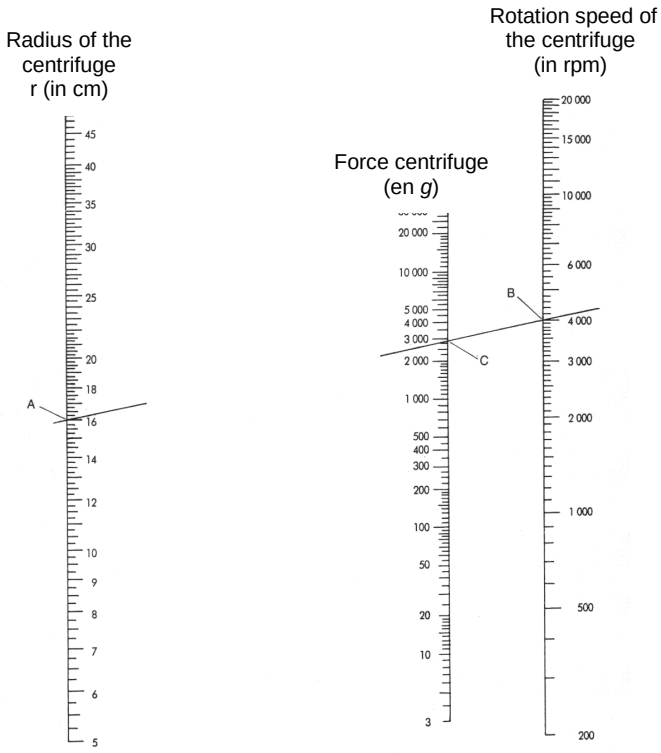
ANNEX 10

Relation rotation speed of a centrifuge and the centrifugal power



Relation between centrifugal (*g*) and the number of rotations per minute (rpm) in function of the radius of the rotor of the centrifuge (r). Combining the two values r and *g* with a line, an intersection is obtained with the line of the rotation speed of the centrifuge, the value in rpm to use for this centrifuge and this rotor. The relation formulae between g and rpm is :

$g = 0.00001118 \times r \times rpm^2$. (N.B. : the radius of the centrifuge is expressed in cm)

ANNEX 11

Useful internet sites

World Health Organization in French, English and Spanish (documents can be downloaded from blood transfusion):

http://www.who.int/topics/blood_transfusion/fr/

International Society of blood transfusion in French and English

http://www.isbt-web.org/

Public Health Agency (Canada) in French and English :

http://www.phac-aspc.gc.ca/hcai-iamss/tti-it/risks_f.html#tab2

Site of the Canadian Society of blood in French and English :

http://www.medecinetransfusionnelle.ca/

Site de l'hémovigilance en français :

http://www.hemovigilance.org/

Site français de l'Institut National de transfusion sanguine (INTS) en français :

www.ints.fr

Site du service du sang de la croix rouge de Belgique en français :

http://www.transfusion.be/

GLOSSARY

Agglutination: The clumping together of cells

Agglutinins : antibodies which provoke agglutination of the red blood cells. It always concerns IgM.

Allo antibodies : (= iso antibodies) antibodies form against « foreign » antigens.

Alloimmunization: The immune response in which an antibody is produced when a body meets a foreign antigen.

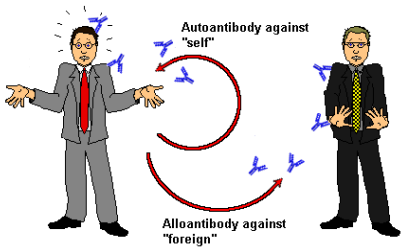
Antibody : specialized proteins (immunoglobulins) which bind specifically and react with the antigen. As a rule, antibodies to an antigen are only formed if the corresponding antigen is missing in the antibody forming organism. One Immunoglobulin will react only with a specific antigen (specificity). There are 5 classes : IgM, IgG, IgA, IgE, IgD.

Antibodies (warm-): (IgG) antibodies with a maximal activity at 37 °C.

Antibodies (cold-): (IgM) antibodies with a maximal activity at 4°C.

Antigen : A substance which is recognized as foreign by a living organism and as a result induces a specific immune response. Every molecule recognized by the immune system is described as an antigen. This predominantly concerns proteins which occur either in the pure form or combined with other substances.

Auto antibodies : Antibody form against « own » antigens.



Autologous transfusion: the transfusion of any component that was donated by the patient himself, his own blood or blood component.

Blood donor (familial -): a donor who gives blood when it is required for family member

Blood donor (voluntary-non-renumerated): altruistic blood donor, who is not compensated for it in any way.

Blood donor (regular, volontary-): regular altruistic blood donor, who is not compensated for it in any way. Regular: at least three times donated and in general at least once a year.

Blood donor (Remunerated-): a person who is selling his blood.

Blood donor (Replacement-): familial blood donor in the context of a blood bank. If a blood bank doesn't have enough voluntary blood donors, they can ask the family of a transfused patient to replace one or more transfused blood units. In this case, it is not the "familial" blood that is given to the patient, but a bag of stored blood. This type op donor permits to "reconstitute" the blood stock in the context of a non self sufficient blood bank with voluntary, non remunerated donations.

Blood grouping (forward): a test to detect ABO antigens on the surface of red blood cells of a patient using antibodies (antiserum) specific to ABO group.

Blood grouping (reverse): a test to detect ABO antibodies in the serum or plasma of a patient using known red blood cells.

Centrifuge for blood bank : Rotor, tubes and speed adapted for wash of red blood cells and for centrifugation for reading of agglutinations. Examples : model Immufuge-II de Baxter©, model Diacent-12 de Diamed©. There also exist automatic red blood cell washers of the type Diacent-CW de Diamed©. These washers permit to make the work easier and to reduce duration compatibility test, at the price of a moderate fragile instrument..


Diacent -cellwash

Diacent -12

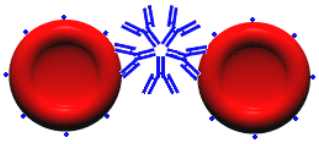
Immufuge II

Coagulation: Clotting of blood which takes place when blood is collected into a dry container or reaches an open wound.

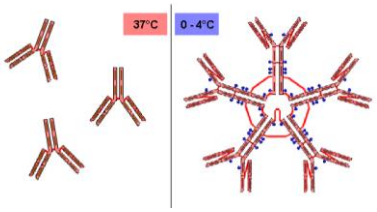
Cold agglutinins : antibodies found in some persons. These antibodies agglutinate the own red blood cells of the subject, but only at low temperature (maximal agglutination at 4° C, weaker agglutination at 22°C). These antibodies are not active at 37°C. It always concerns about IgM. They are meanly directed against l and i antigens.

Complete antibodies : (also called agglutinins) Some antibodies, of the **IgM** class, can agglutinate erythrocytes directly in a saline medium They are cold antibodies with a maximal activity at 4 °C.

IgM



IgG **IgM**



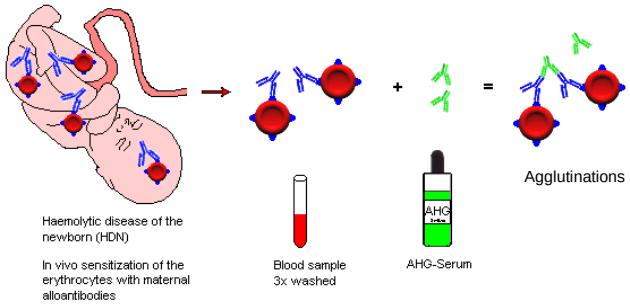
Confidential self-exclusion: The removal and disposal of a unit of blood after the donation at the request of the donor.

Coombs serum : anti-human globulin antibodies. Obtained by immunisation of animals (rabbit or goat). The Coombs serum can be **polyvalent** (directed against all human immunoglobulins : IgM, IgG, IgA,…) or **mono specific** (directed against just one specific human antibody).

(Rapid) cross-matching: a term used when testing before transfusion the patient's serum (or blood in case of rapid cross matching) against the donor's red cells and the donor's serum against the patient's red cells (the latter is not done during a rapid cross match).

Cross-reaction: when an antibody recognizes not only its corresponding specific antigen, but also other antigens that may have certain similarities

Direct Coombs Test (or : direct anti-globulin test) : Test using the Coombs serum, permitting to demonstrate non agglutinating antibodies (IgG) *in vivo* fixed on the red blood cells. This test permits for instance determination of maternal allo antibodies fixed on the red blood cells of the newborn or of the fœtus (p.i. in case rhesus incompatibility).



Direct donation: a donation that is given specifically for transfusion to a named patient.

Globulins: plasmatic proteins containing the antibodies or the immunoglobulins.

Haemolysin : substance that is destroying the red blood cells. Generally it concerns antibodies, but this expression is also used for other substances (p.i. the continence of the venin of certain serpents).

Haemolysis: the breaking down (destruction) of the red cell membrane, liberating the cell's contents.

Haemolytic disease of the new-born: a disease in which maternal antibodies cross the placenta and destroy the foetal red cells that possess the corresponding antigen, leading to anaemia.

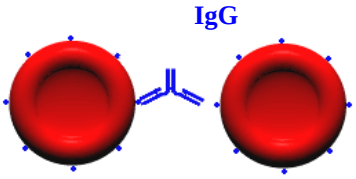
HLA : Human leukocyte antigens. The HLA system is a human major histocompatibility system. A part located on the human genome from which the genes code especially for the major histocompatibility antigens which intervene in the control of the immune response and in the phenomena of transplantation rejection.

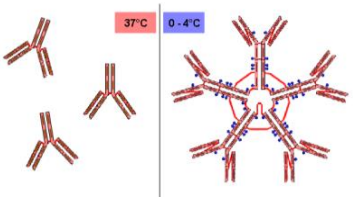
Humoral immunity: Mechanism of defence of an organism, involving antibodies.

Indirect Coombs Test (or : indirect anti-globulin test) : Test using the Coombs serum, permitting to demonstrate the presence of non agglutinating antibodies in serum (IgG). It is used p.i. for the major compatibility test.

Incomplete antibodies (**IgG)** : their fixation on the membrane of the red blood cells is not sufficient to provoke agglutination. These are warm antibodies, with a maximal activity at 37°C. Their detection is based upon the use of artificial techniques which permit to bring red blood cells together.
This is provided for example by:

- bovine albumin
- photolytic enzymes
- Coombs serum.





Irregular antibodies: appearing after immunisation, in certain subjects, when the corresponding antigen does not exist on the surface of their red blood cells.

Iso antibodies: antibodies developed by an organism, as an answer to an antigen from another individual of the same species. (synonym for Allo antibody)

LISS albumin medium: solution with Low Ionic Strength, enhancing the fixation of antibodies on the erythrocytes, associated with macromolecules (albumins) which increase the dielectric constant of the medium (decreasing so the rejection of the red blood cells). This medium increases thus the

possibilities of the reactions between red blood cells and antibodies. It is associated with the Coombs test and permits the demonstration of irregular, incomplete antibodies of type IgG.

Living blood bank: registered group of people living nearby the hospital, with a known blood group, which accept to donate freely their blood in case of need.

Monoclonal antibodies : Very specific antibody products from one single cell or identical precursor of this cell, directed against a specific epitope of an antigen. (As the opposite of polyclonal antibodies)

Natural antibodies : Antibodies found in the serum without apparent pre immunisation from the corresponding antigen. They appear apparently spontaneously. If this is not the case, they are called immune antibodies.

Packed cells: blood bag, containing mainly red blood cells, obtained after elimination of the plasma (and the white blood cells).

Prevalence: The proportion of a specific population that is infected with the infectious agent at a particular time.

Primary antibody response: The immune response that the body makes when meeting a foreign antigen for the first time.

Regular antibodies : antibodies present in all subjects in absence of the corresponding antigen. (As the opposite of irregular antibodies). Irregular antibodies occur also naturally, but not in all subjects.

Risk behaviour: behaviour that exposes a person to the risk of acquiring transfusion-transmissible infections.

Rouleaux agglutination: also pseudo-agglutination, a false agglutination, which is usually due to an upset in the albumin/globulin ratio, characterized by a piling up of the blood cells, resembling piles of coins. Often more pronounced in pregnancy and severe anaemia,

Saline medium : physiological medium that contains 9 g sodium chloride (NaCl) per litre of distilled water. This is an isotonic solution that permits to preserve the cell volume.

Secondary antibody response: The increase in titre of an antibody when meeting its antigen for the second time.

Sensitized cell: a cell coated with antibody, but not agglutinated.

Seroconversion: a change in serostatus of an individual from seronegative to seropositive.

Seroprevalence: The proportion of a specific population testing seropositive for the infectious agent at a particular time.

Transfusion of full blood: transfusion of a blood bag, containing red blood cells, white blood cells and plasma. As an opposite of fractioned blood, permitting to transfuse packed cells (erythrocytes), plasma, etc.

Transfusion (Warm): transfusion of a blood bag immediately after the taking, without storing in a refrigerator. This system is meanly applied for familial blood donors.

Transfusion (Cold): Transfusion of a blood bag that has been stored in the fridge. This system is applied with voluntary blood donors and blood bank.

Transfusion-transmissible infection: An infection that is capable of being transmitted by blood transfusion.

Window period: The period between infection and the first appearance of a circulating detectable marker of that infection.