**Lec:2**

**SPECIAL STAINS**

**1-Modified Acid-Fast Staining Procedure**

This technique is useful for the identification of oocysts of the coccidian species (*Cryptosporidium*, *Cystoisospora*, and *Cyclospora*), which may be difficult to detect with routine stains such as trichrome. Unlike the Ziehl-Neelsen Modified Acid-Fast Stain, this stain does not require the heating of reagents for staining.

**Specimen:**

Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid, bile, pulmonary samples (induced sputum, bronchial wash, biopsies) may also be stained.

**2-Quick-Hot Gram-Chromotrope Staining Procedure**

This is an alternative stain to the chromotrope procedure that is a fast, reliable, and simple method of staining smears to demonstrate microsporidian spores in fecal and other clinical specimens.

**Specimen:**

Prepare a thin smear of the material to be stained (such as feces, urine, sputum, saliva, and cell culture supernatant) and air dry.

**3-Modified Safranin Technique (Hot Method) Staining Procedure**

For *Cyclospora*, *Cryptosporidia*, and *Cystoisospora* species:

Oocysts of *Cyclospora* in clinical specimens are routinely demonstrated using modified acid-fast stain (cold). However, with that technique, the oocysts stain variably from nonstaining to full staining leading to possible misidentification. The modified safranin technique produces a more uniform staining of these oocysts. The stain needs to be heated to boiling using either a hot plate or microwave.

**Specimen:**

Concentrated sediment of fresh or formalin-preserved stool may be used. Other types of clinical specimens such as duodenal fluid may also be stained.

**4-Trichrome Staining Procedure**

It is generally recognized that stained fecal films are the single most productive means of stool examination for intestinal protozoa. The permanent stained smear facilitates detection and identification of cysts and trophozoites and affords a permanent record of the protozoa encountered. Small protozoa, missed by wet mount examinations (of either unconcentrated or concentrated samples) are often seen on the stained smear.. It is a rapid, simple procedure, which produces uniformly well-stained smears of the intestinal protozoa, human cells, yeast, and artifact material.

**Specimen:**

The specimens usually consist of fresh stool or stool fixed in polyvinyl alcohol (PVA) smeared on microscope slides and allowed to air dry or dry on a slide warmer at 60°C. Stool preserved in sodium acetate-acetic acid-formalin (SAF) or some of the one-vial fixatives can also be used.

**5-Calcofluor White Staining Procedure**

This chemofluorescent technique is useful for the detection of microsporidia, *Acanthamoeba* spp., *Pneumocystis jiroveci*, and *Dirofilaria* spp.

**Specimen:**

Prepare a thin smear using approximately 10 µl of fresh or preserved specimens on a glass slide. Specimens may include stool, urine, culture or other types of samples. Heat fix on a slide warmer at 60°C until completely dry (5-10 minutes).

6- **Leishman's stain**, is used in [microscopy](https://en.wikipedia.org/wiki/Microscopy) for [staining](https://en.wikipedia.org/wiki/Staining) [blood smears](https://en.wikipedia.org/wiki/Blood_smear). It provides excellent stain quality. It is generally used to differentiate and identify [leucocytes](https://en.wikipedia.org/wiki/Leucocyte), [malaria](https://en.wikipedia.org/wiki/Malaria) parasites, and [trypanosomas](https://en.wikipedia.org/wiki/Trypanosoma%22%20%5Co%20%22Trypanosoma).

7- **methylene blue** 8**-fluorochrome-stained parasites 9-acridine orange staining**

 **10 -wright's  stain**  a mixture  of  eosin  and  methylene  blue,  used  for  demonstrating  blood cells and malarial parasites.

11- **hematoxylin  and  eosin  stain**  a mixture  of hematoxylin  in distilled  water  and  aqueous  eosin  solution , employed universally  for  routine  examination  of tissues.

 **Kato technique** (also called the **Kato-Katz technique**) is a laboratory method for preparing [human stool](https://en.wikipedia.org/wiki/Human_feces) samples prior to searching for parasite eggs The Kato technique is now most commonly used for detecting [schistosome](https://en.wikipedia.org/wiki/Schistosoma%22%20%5Co%20%22Schistosoma) eggs. It has in the past been used for other [helminth](https://en.wikipedia.org/wiki/Helminth%22%20%5Co%20%22Helminth) eggs as well. It cannot be used to identify [hookworm](https://en.wikipedia.org/wiki/Hookworm) eggs because they collapse within 30 to 60 minutes of preparation using this method. The other main argument against the Kato technique is that it is messy and therefore exposes technicians to an unnecessarily high risk of infection.

**-A fecal Baermann** Technique**:** is a special test that detects certain types of parasites or "worms." Many parasites shed their eggs in the host's stool. Infection with these parasites can be diagnosed with a simple “fecal flotation” test in which the stool is examined microscopically for the presence of parasite eggs. However, some parasites pass free larvae instead of eggs in the stool. These larvae cannot be detected by routine fecal flotation, and a special technique called a **“fecal Baermann”**is needed to diagnose infection.

Stool material is placed in a special funnel-shaped collection device and covered with warm water. The sample is left to stand for 1-2 hours or longer. During this time, larvae actively move out of the stool material and sink to the bottom of the funnel where they are collected and identified.

* 

**In vitro cultivation:**

* + **Xenic cultures:**Culture of parasites grown in association with an unknown microbiota (i.e. microbial population)Cultivation of *E histolytica*
	+ **Monoxenic cultures**:Parasite grown in association with a single known bacterium Acanthomoeba culture from corneal biopsy with *E coli*
	+ **Axenic cultures:**Pure culture without bacterial associate*Leishmania* culture

**Other methods of cultivation**

 -Animal inoculation-

-Xenodiagnosis :Use of arthropod host as an indicator of infection

* + Reduvid bug in the diagnosis of Chagas’ disease

**When do we culture parasites**

Obtain definitive identification

* + -Repeated microscopy fails , indirect evidence

 (e.g., antibody response) not conclusive

-Test antimicrobial susceptibility

-Measure response to treatment

-Characterize the agent

-Strain bank for future use

-Vaccine development, antigen production, research

Rapid antigen detection tests are increasingly becoming useful



## GENERAL PRINCIPLES

Although the province of parasitic cultivation is very diverse, there are certain principles which are applicable at large to the subject:

1. Parasitic helminths are more difficult to cultivate than protozoa. The complexity of helminth body configuration and metabolism, and inability to meet essential environmental conditions account for failure to complete their life-cycles under artificial conditions
2. Cell cultures are used for the obligate intracellular parasites, for example Plasmodium spp. and coccidian.
3. Various kinds of nutrients such as blood, serum, haem, egg, peptone, minerals and carbohydrates are used in the culture media.
4. The temperature required for optimum growth is usually 37°C though lower temperatures may be required in few cases, e.g. 25°C for  Leishmania promastigotes.
5. Incubation condition is aerobic with some exceptions like microaerophilic conditions for amoebae and Giardia and 5% CO2 for Plasmodium spp.
6. Identification tools include parasite's characteristic morphology, direct fluorescent antibody assay, polymerase chain reaction, enzyme immunoassay, etc

### Luminal parasitic protists

Luminal protists are first grown in xenic cultures, gradually weaned, then isolated in axenic cultures. While T. vaginalis and Giardia intestinalis can be established directly into axenic cultures, E. histolytica and Blastocystis hominis have never been grown axenically without first being established in xenic cultures. Dientamoeba fragilis and Balantidium coli have never been grown successfully in axenic culture to the best our knowledge.Some of the important media used for cultivating luminal parasitic



 **Xenic Culture Media**

#### 1-Rice starch.

Purified rice starch is important for growth of E. histolytica in all the following media. Entamoeba will not ingest all forms of rice. Most important is the size of the rice particle, as it must be within the ameba's ability to phagocytize it. A source for a very reliable rice starch is listed below under “Sources of Medium Materials.”

#### 2- media.

#### (i) LE medium.

To prepare LE medium (NIH modification of Boeck and Drbohlav's medium

#### (ii) Robinson's medium.

Robinson's medium is a complex medium that has nevertheless found widespread use for the isolation of enteric amebae.

#### 3-Monophasic media.

#### (i) TYSGM-9.

####  (ii) Robinson's medium.

The liquid overlay from Robinson's medium ([54](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC118080/#r54)) as described above can be used as a monophasic growth medium. This is especially useful once isolates are established in culture.

### Axenic Culture Media

One constant problem facing those who rely on axenic cultures is the fastidiousness of these organisms. Although the others are also affected to a significant degree, this is especially true of E. histolytica. Lot-to-lot variations in several components of the axenic culture media in particular can have profound effects on the ability of a medium to support growth of the organisms; some lots may even be toxic. Trypticase (casein digest peptone), yeast extract, and serum are the medium components most commonly affected, but the quality of the distilled water and even the type of glass used in making the culture tubes can cause problems (screw-cap borosilicate glass tubes should be used when possible). For this reason, we highly recommend that those wishing to undertake axenic cultivation of these organisms test the ability of each new lot of reagent to support growth before starting to use it.

#### *E. histolytica*. (i) TYI-S-33.

####  (ii) YI-S.

####  (iii) LYI-S-2.

#### *G. intestinalis*. (i) TYI-S-33 (Keister's modification).

####  (ii) YI-S.

#### *T. vaginalis*. (i) TYM.

####  (ii) Hollander's modification of TYM.

####  (iii) TYI-S-33 and YI-S.

#### *B. hominis*. (i) LE medium.

####  (ii) IMDM.

### Maintenance of cultures

Established cultures of all parasites are handled largely in the same way. Xenic cultures of E. histolytica, D. Fragilis, B. hominis and B. coli are routinely passaged at 48–72 h intervals. More inoculum should be used when incubating for longer duration than that for shorter incubation periods. Xenic cultures should be passaged using two or more inoculum sizes to ensure a successful subculture. Established axenic cultures of T. vaginalis, E. histolytica, G. intestinalis, and B. hominis are passaged at 72 and 96 h intervals. For doing subculture, cultures are chilled in an ice-water bath for 5 min (xenic and axenic cultures of E. histolytica) or 10 min (G. intestinalis) to release trophozoites attached to the glass culture tube. In T. vaginalis, B. coli, and B. hominiscultures, most organisms will be nonadherent and the tubes need not be chilled unless an accurate count is desired. Tubes are inverted several times to disperse the cells and a measured inoculum is passed aseptically to a culture tube containing fresh medium. The tubes are capped tightly and incubated at 37°C, either vertically (xenic cultures of T. vaginalis, and axenic cultures of B. hominis) or at 5° to the horizontal (established axenic cultures of E. histolytica and G. intestinalis). For axenic B. hominis cultures the medium must be pre-reduced for 48 h before inoculation in an anaerobic jar.



In cases of leishmaniasis, blood samples, needle aspirates, and punch or deep organ biopsies can be used for culture. the hemoculture positivity was found to be 67%, with average growing time of 10 days.

Leishmania promastigotes are cultured at temperatures below 28°C.NNN medium, first used for isolation of the agent of oriental sore by Nicolle

Coccidia and microsporidia



### Helminths

Attempts have been made to culture parasitic helminths in nutrient media, and in some instances partial success has been achieved. Some common media used for helminth cultivation are shown in [Table 6](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4166808/table/T6/). Aseptic techniques in bacteria-free media have prolonged the life of the worms, and in some species have resulted in development from larva to a more mature sexual stage or to sexual maturity. The complexity of helminth metabolism and inability to meet essential environmental conditions account for failure to complete the life-cycles of many of these organisms under artificial conditions



## MAINTENANCE OF STOCK CULTURES

There are various methods for maintaining stock cultures of protozoan parasites, for example repeated subcultures, freezing at −20°C and ultra-low temperature freezing at −70°C to −196°C using cryoprotectants such as 10-30% glycerol, 5% dimethyl sulfoxide and sucrose.They can also be stored as dried filter paper strips at room temperature

## iN VIVO CULTIVATION OF PARASITES

Some animal parasites which have thus far been refractory to in vitro cultivation may be developed in appropriate animal tissues.

## XENODIAGNOSIS

Triatomid bugs have been employed for diagnosing Trypanosoma cruzi infections where it has not been possible to demonstrate the organisms in blood films. Laboratory reared bugs are fed upon patient's blood, and the intestinal contents of the bugs are examined for flagellates 10-30 days after the blood meal. These may then be inoculated into mice for further confirmation.

**FECAL FLOTATION**

 Non-centrifugation flotation procedures perform too poorly to be recommended; centrifugation flotation procedures are clearly superior. Centrifugation procedures are quicker to perform, enable you to detect more types of parasites, and you will be able to diagnose and effectively treat parasitic conditions more frequently, thereby increasing financial benefit for both your client and your practice.

**1. Saturated salt solution:**,General purpose solution.

**2. Salt/sugar solution:** General purpose solution.

**3. Sodium nitrate:** This solution is sometimes used for strongyleoides eggs.

**4- Saturated sugar solution:** This solution should be used if the eggs are required for culturing as it has little effect on their viability.

**5-Modified Sheather’s Flotation Solution**

6-- **Zinc Sulfate Centrifugal Flotation Technique**

**Note:-Ethyl acetate sedimentation**

