rim of **cyanophilic/eosinophilic cytoplasm** and eccentric nuclei.

- Central cytoplasmic brown/yellow staining indicates glycogen production.
- They are seen with **high progesterone levels** when superficial squamous cells exfoliate more readily: thus, they may be **seen with progesterone-only contraceptive use.**

Uses of Pap smears:

- Pap smears are used for hormonal evaluation to categorising their abnormalities into non-neoplastic, benign, and malignant types.

A) **Assessment of hormonal status:**

- It is **best** performed on samples taken from **lateral vaginal smears.**
- Ideally, at least **3 smears** obtained on **alternate days** for evaluation.
- Following Indices are used for description of cytohormonal patterns:

1) **Pyknotic index (PI)**:
   - The percentage of cells having small, dark, shrunken nuclei (**less than 6** µm in size).
2) **Acidophilic index (AI):**
   - The relative **proportion** of cells containing **acidophilic (pink)** and **basophilic (blue) cytoplasm**.
3) **Maturation index (MI):**
   - Most widely used method.
   - One hundred squamous cells are counted and grouped according to their type—parabasal, intermediate, or superficial.

B) **Inflammations**

1) **Trichomonas Vaginalis:**

- This is an **oval or pear-shaped** organism that varies from 8 to 30 micro meter **(Image 21.2).**

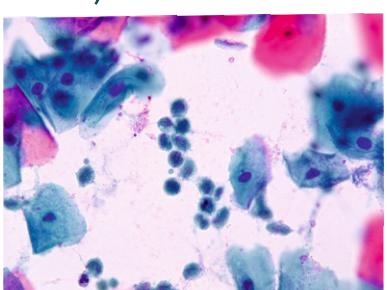


Image 21.2: Trichomonas vaginalis : Oval or pear-shaped organism with **thin,** elliptical nucleus must be identified to diagnose this infection.

- The trichomonas **nucleus (thin, elliptical)** must be identified to diagnose this infection.
- **Red granules** in cytoplasm may be seen.
- Slightly enlarged, dark nuclei and perinuclear halos are common, mimicking low grade dysplasia.

2) **Leptothrix:**

- It is **mixed lactobacilli.**
- The organisms are long, thin (less than **half as thick as Candida**) and flexible **(Image 21.3).**

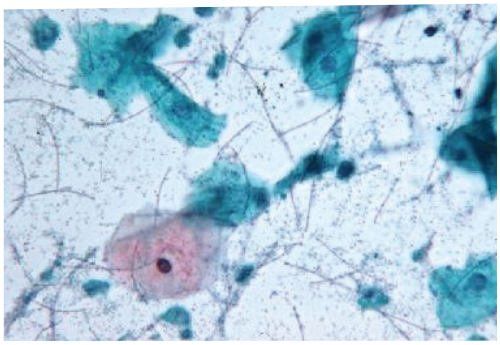


Image 21.3: Leptothrix and trichomonas: Organisms are long, thin (less than **half as thick as Candida)** and flexible. **If leptothrix is present, Trichomonas is usually present,** but reverse is not true.

- **If leptothrix is present, Trichomonas is usually present**, but reverse is not true.

3) **Candida Species:**

- It is associated with a **change in vaginal glycogen flora or pH**.
- For example: Pregnancy, late luteal phase of cycle, diabetes mellitus, immunosuppression, debilitating disease, steroids, birth control pills, broad spectrum antibiotics, chemotherapy are associated with candida infection.
- **Pseudohyphae (sticks**) and **yeast (stones**) are seen**. (Image 21.4)**

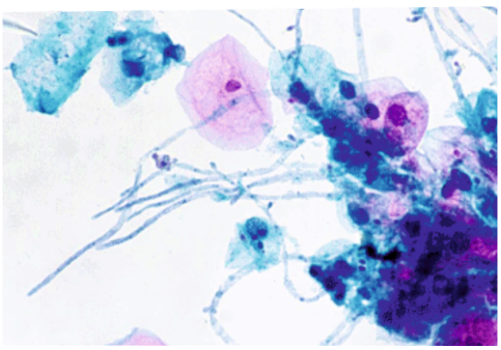


Image 21.4: Candida : Pseudohyphae (sticks) and yeast (stones) are seen.

4) **Actinomyces**:
- It is **associated with IUD use**; rarely is associated with other **foreign objects (tampons or pessaries).**
- The patient may be asymptomatic or have pelvic pain.
- Cytologic findings are **colonies** of variably **gram-positive**, long, thin, **filamentous bacteria** that are reddish, branch are irregularly beaded, and **radiate from central area (Image 21.5).**

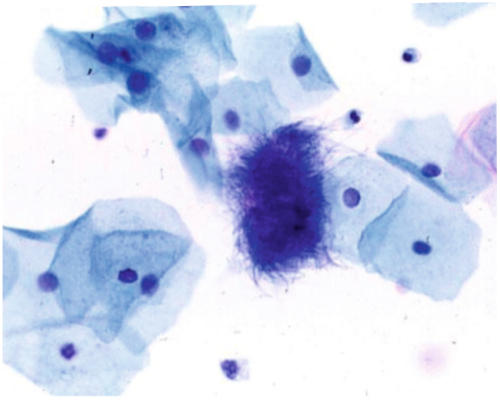


Image 21.5: Actinomyces : Colonies of variably gram-positive, long, thin, filamentous bacteria that are reddish, branch are irregularly beaded, and radiate from central area.

5) **Gardnerella vaginalis (Bacterial Vaginosis):**
- It is a **gram-negative**, comma-shaped coccobacillus.
- Smears usually shows characteristic **granular blue small coccobacilli** covering and hiding squamous cells **(clue cells) (Image 21.6).**

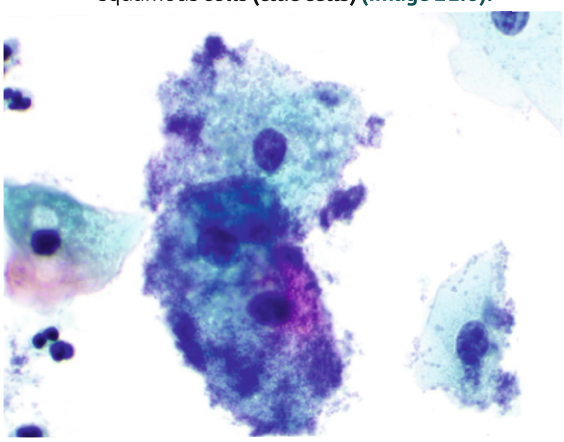


Image 21.6: Bacterial Vaginosis (Clue cells) : Smears usually shows characteristic **granular blue small** coccobacilli covering and hiding squamous cells **(clue cells).**

6) **Chlamydia infections:**
- Chlamydia trachomatis is a tiny, **gram-negative** bacterium that exists in two different forms:
  a) **Elementary body**, which is the **infectious form**, and
  b) **Reticulate body**, which is the **replicative form.**
- Chlamydial organisms are **large, glassy inclusions** containing both reticulate bodies and elementary bodies are clearly visible **within squamous epithelial cells (Image 21.7).**

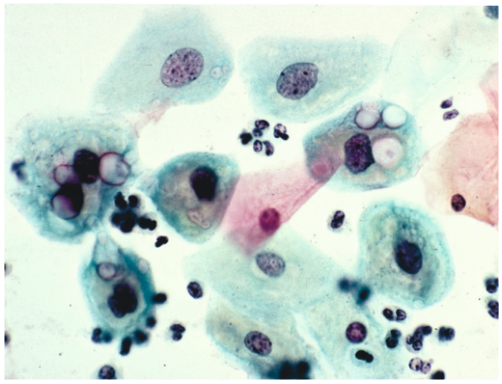


Image 21.7: Chlamydia : Chlamydial organisms are **large, glassy inclusions** containing both reticulate bodies and elementary bodies are clearly visible **within squamous epithelial cells.**

7) **Koilocytes :**
- It is a **squamous epithelial cell** that has undergone a number of structural changes, which occur as a result of infection of the cell by **human papillomavirus.**
- **Koilocytosis or koilocytic atypia** or koilocytotic atypia are terms used in histology and cytology to describe the presence of koilocytes in a specimen.
- Koilocytes may have the following cellular changes **(Image 21.8):**

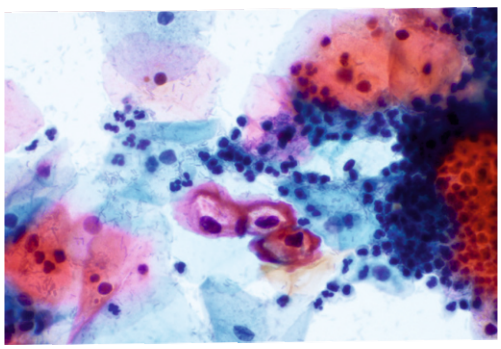


Image 21.8: Koilocytes : Showing **Nuclear enlargement** (two to three times normal size), **Irregularity** of the nuclear membrane contour, **Darker** than **normal** staining pattern in the nucleus **(Hyperchromasia)**, and a clear area around the nucleus, known as a **perinuclear halo.**

a) **Nuclear enlargement** (two to three times normal size)
b) **Irregularity** of the nuclear membrane contour
c) **Darker** than normal staining pattern in the **nucleus**, known as **Hyperchromasia**
d) A clear area around the nucleus, known as a **perinuclear halo.**

High Yield Info.....

❖ **LIQUID-BASED CYTOLOGY (LBC) PREPARATIO NS:**
- It is a special technique for preparation of gynaecologic and non-gynaecologic samples which has following advantage:
  a) **Uniform monolayered dispersion of cells** on smears.
  b) **No overlapping** or **clump formation**.
  c) **No background** material or debris.
- It is a pre-requisite for quantitative analysis and automated devices.

❖ **Ideal fixative** for routine cytological examination is **Papanicolaou's fixative** (equal parts of **ether** and **95% ethanol).**

Immunofluorescence (IF) or Cell Imaging:

- Basic principle of this method is to use of **antibodies** to label a specific target antigen with a **fluorescent dye** (also called **fluorophores or fluorochromes).**
- Most commonly used fluorophores are **fluorescein isothiocyanate (FITC).**
- Antibodies are chemically conjugated to fluorophores.
- Fluorophore allows visualization of the target distribution in the sample under a fluorescent microscope (e.g. epifluorescence and confocal microscopes).
- It is of **two types:**

A) Direct (Primary) Immunofluorescence.
- It uses a **single antibody** that is **directly** attached to a **fluorophore.**
- Antibody binds to the target molecule, and fluorophore it carries can be detected via microscopy.
- **Direct immunofluorescence** is **less sensitive** than **indirect** immunofluorescence because number of fluorescent molecules that can be bound to the primary antibody is limited.
- It can be valuable tool for the diagnosis of groups of diseases, that are often **difficult to separate clinically** and those cases that are **histologically similar** like **pemphigus, pemphigoid, lupus erythematosus, lichen planus, erythema multiforme, linear IgA disease, epidermolysis bullosa acquista**

B) Indirect (Secondary) Immunofluorescence.
- It uses two antibodies;
  a) **Unlabelled first (primary) antibody** specifically binds the target molecule, and
  b) **Secondary antibody**, which carries the **fluorophore**, recognises the primary antibody and binds to it.
- This is the 2 stage procedure for **in vitro** demonstration of circulating antibodies in the **patient's serum** e.g, **ANA of SLE , Renal lesions like Good pasture syndrome and PSGN (Image 21.9 (A; Goodpasture syndrome** with linear immunofluorescence pattern of **IgG antibodies)** and **(B; PSGN** with **granular pattern** due to formation of **immune complexes**).

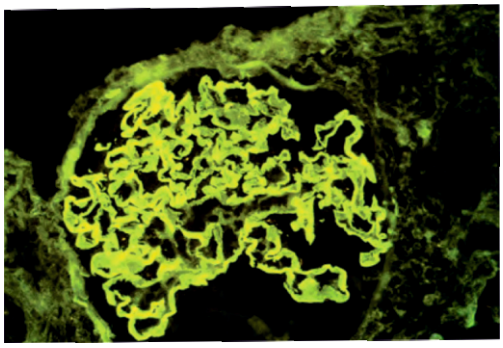


Image 21.9: a) Goodpasure syndrome (Linear IFL)

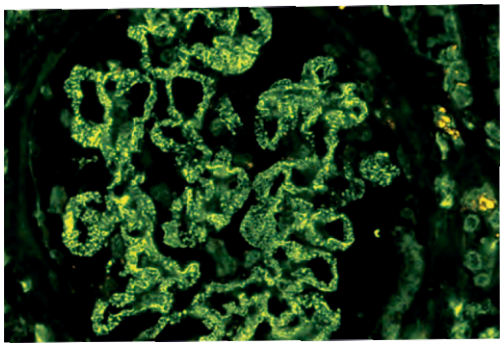


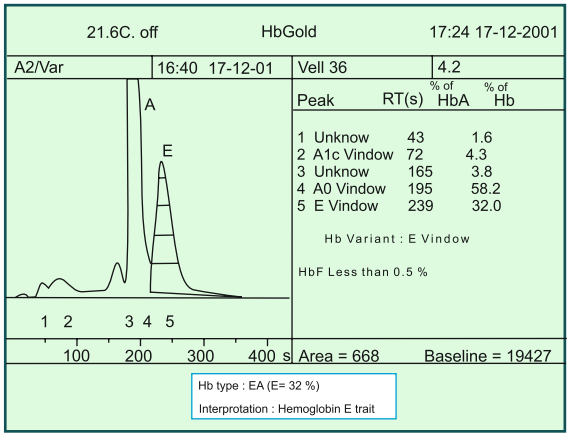
Image 21.9: b) PSGG (Granular IFL)

High Performance Liquid Chromatography (HPLC)

- It has also been referred to as High Pressure Liquid Chromatography.
- **Principle of chromatography**
  - Chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition.
  - This involves:
    A) S**tationary phase** (a solid, or a liquid supported on a solid), and
    B) **Mobile phase** (a liquid or a gas).
  - The **mobile phase** fl ows **through the stationary phase** and carries the components of the mixture with it.
  - **Sample components** that display **stronger interactions** with the **stationary phase** will **move more slowly** through the column than components with weaker interactions.
  - This difference in rates cause the separation of various components.
  - Chromatographic separations can be carried out using a variety of **stationary phases**, including
    1) Immobilized silica on glass plates (**thin-layer chromatography)**
    2) Volatile gases (**gas chromatography**)
    3) Paper (**paper chromatography**)
    4) Liquids (**liquid chromatography**).

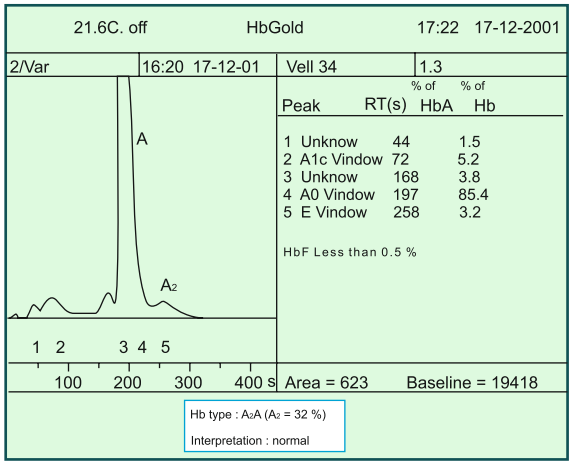
HPLC (principle):

- High performance liquid chromatography (HPLC) is basically a highly-improved form of column **liquid chromatography**.
- Instead of a solvent being allowed to drip through a column under gravity, it is forced through under **high pressures of up to 400 atmospheres.**That makes it much faster.
- All chromatographic separations, including HPLC operate under the same **basic principle**; **separation of a sample** into its constituent parts because of the **difference in the relative affi nities** of different molecules for the mobile phase and the stationary phase used in the separation.
- Separated components are detected at the exit of this tube (column) by a **fl ow-through device (detector)** that measures their amount. An output from this detector is called a "**liquid chroma-togram".**
- Adsorbed **positively charged haemoglobin** molecules are eluted from column into the liquid phase at a rate related to their affi nity for the stationary phase.
- Hemoglobins are provisionally **identifi ed by their retention time** and **quantifi ed by** computing the **area under the corresponding peak in the elution profi le** (Image 21.10 (A) and (B).



Hb type : A_2A (A2=3.2%)

Interpretation : Normal



Hb type : EA (A2=32%)

Interpretation : Hemoglobin E trait

Image 21.10: a) HPLC

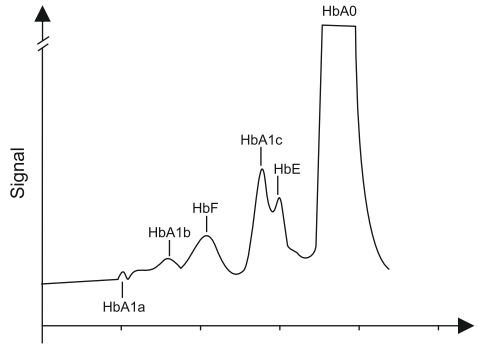


Image 21.10: b) HPLC 1

- **Chromatogram** is a graph for **Time scale Vs Absorbance** (at column Adsorption and at detector Absorption).
- **Limitation** of HPLC is **HbE, Hb-Lepore** co-elute with **HbA2.**

Cellulose Acetate Electrophoresis

Principle:

- Electrophoresis is the movement of charged particles in an electrical field.
- **Haemoglobin** is **negatively charged at pH 8.4 to 8.6** and **migrates toward the anode** in an electric field with **cellulose acetate** as the support medium.
- During electrophoresis, many haemoglobin variants are separated on the basis of **different charges** due to alteration in their primary structure.
- This separation allows **preliminary identification** of these **haemoglobins (Image 21.11).**

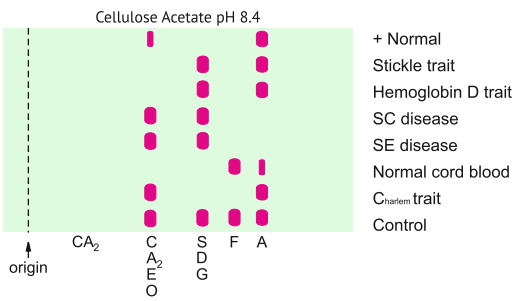


Image 21.11: Cellulose gel Electrophoresis

Capillary Electrophoresis

- Capillary electrophoresis separates hemoglobin variants by **electroosmotic flow** and **electrophoretic mobility** in alkaline buffer (pH 9.4).
- Multiple samples (two to eight, depending on the instrument used) undergo high-resolution separation concurrently in **silica glass capillaries**, taking **approximately eight minutes** to complete the analysis.
- For Hb variant detection, **UV at 415 nm wavelength** is used.
- The detection methodology is similar to one used in HPLC systems. As a result, the methodology is sometimes considered a "**hybrid**" type of separation technique between **classical zone electrophoresis and liquid chromatography.**
- **Advantage over hPlc: i**t can isolate HbA2 and HbE, whereas **hPlc** will report **same position for both hba2 and hbE (image 21.12).**

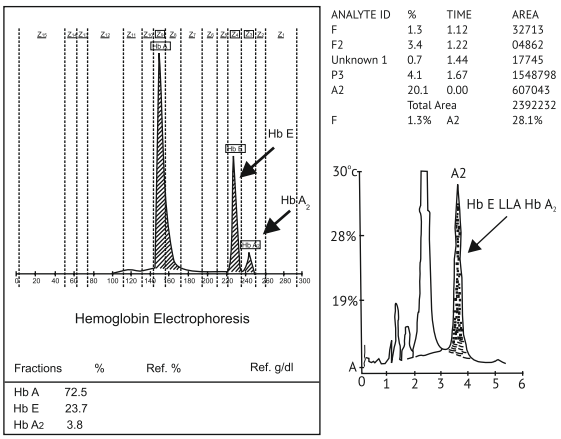


Image 21.12: Capillary electrophoresis vs HPLC

Fluorescence In Situ Hybridization (FISH):

- FISH is a molecular cytogenetic technique that **can detect chromosomal abnormalities** that cannot be appreciated by standard chromosomal analysis (e.g. **microdeletion syndromes**) or when mitotic cells are not available for chromosomal analysis (e.g. X/Y FISH for cross-sex transplants).
- Briefl y, **metaphase chromosomes** or **interphase nuclei** are denatured on the slide, as is the fl uorescently labeled DNA probe.
- **Probe and chromosomes/nuclei are then hybridized**, slides are washed, counterstained and analyzed by fl uorescent microscopy.
- Types of FISH probes are:
  a) Unique sequence probes (e.g. microdeletion syndromes)
  b) Whole chromosome painting probes
  c) Repetitive probes (e.g. centromeric alpha satellite probes, subtelomeric probes)