YEDITEPE UNIVERSITY
SCHOOL OF MEDICINE

PHASE II COMMITTEE V
TISSUE DAMAGE and NEOPLASM
LABORATORY PRACTICE SESSIONS
MICROBIOLOGY LABORATORY MANUAL
2016 – 2017

DEPARTMENT OF MICROBIOLOGY AND
CLINICAL MICROBIOLOGY

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**Sub-titles shown in italic will be practiced.*

Acknowledgement: *We'd like to thank to Prof. Dr. Sesin Kocagöz, Prof. Dr. Tanıl Kocagöz, Prof. Dr. Gülden Çelik, and Assoc. Prof. Dr. Yeşim Gürol for their previous contribution to certain parts of this manual.*

1.1. Principles and Procedures of Laboratory Safety

Universal (Standard) Safety Precautions

All healthcare workers should strictly follow these rules in practice

1. Blood and other body fluids from all patients should be considered infective.
2. All specimens of blood and body fluids should be in a well-constructed container with a secure lid to prevent leakage during transport.
3. Care should be taken when collecting each specimen to avoid contaminating the outside of the container or the laboratory form accompanying the specimen.
4. If there is potential for or actual contamination, an outer container should be used (i.e., bagging).
5. All specimens should be labeled with biohazard insignia.
6. All persons processing blood and body fluid specimens (e.g., removing tops from vacuum tubes) should wear gloves. Facial barrier protection should be used if splashes or sprays of blood or body fluids can occur.
7. For routine procedures, such as histologic and pathologic studies, or microbiologic culturing, a biological safety cabinet is not necessary. However, biological safety cabinets (Class I or II) should be used whenever procedures are conducted that have a potential for aerosolization or droplets. These include activities such as blending, sonicating, and vigorous mixing.
8. Mechanical pipetting devices should be used for manipulating all liquids in the laboratory. Mouth pipetting must not be done.
9. To prevent injuries, needles and syringes should be used with caution. Needles must not be recapped, purposely bent, broken by hand, removed from a disposable syringe, or otherwise manipulated.
10. After they are used, all sharps are to be placed in a clearly labeled, puncture-resistant container for transport to disposal sites. Sharps containers should be placed as close to the work site as is practical. To prevent overfilling and resultant accidental skin punctures, sharps containers should be removed as soon as they are filled.
11. Laboratory work surfaces should be decontaminated with an appropriate chemical germicide after a spill of blood or other body fluids and when work activities are completed.
12. Disinfect all countertops with freshly diluted bleach (aqueous sodium hypochloride; 1:10), with 2.5% phenol, or with another effective solution. The period of contact with the diluted bleach solution should be at least 15 minutes. For other commercial disinfectant products, follow manufacturer instructions.
13. Contaminated materials used in laboratory tests should be decontaminated before reprocessing, or placed in bags and disposed of in accordance with institutional policies for disposal of infective waste.

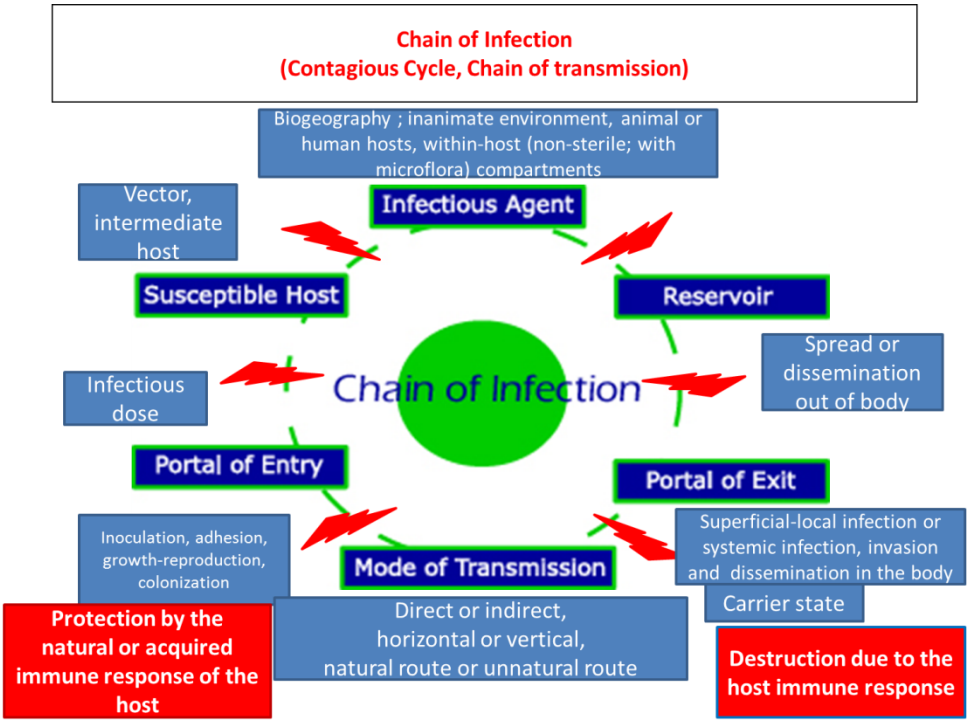
14. Scientific equipment that has been contaminated with blood or other body fluids should be decontaminated and cleaned before being repaired in the laboratory or transported to the manufacturer. Equipment that cannot be completely decontaminated must be labeled with the biohazard insignia.
15. Hands and other skin surfaces must be washed immediately and thoroughly if contact with blood or other body fluids occurs. Hands must be washed immediately after gloves are removed and after completing laboratory activities. All personal protective equipment should be removed before leaving the laboratory.
16. Gloves must be worn when it can be reasonably anticipated that the health care worker will have contact with blood, other potentially infectious materials, mucous membranes, non-intact skin, or when handling contaminated items or surfaces. Gloves must be worn during vascular access procedures, including phlebotomy and finger or heel sticks.
17. Gloves should be replaced as soon as feasible if they become torn, punctured, or lose their ability to function as a barrier. Disposable gloves should not be washed or decontaminated for reuse because this will compromise the impermeability of the material. Gloves should be replaced between patient contacts.
18. Work areas should be covered with absorbent paper. Aerosols must be prevented during pipetting, mixing, shaking, vortexing, and centrifuging operations.
19. To minimize the need for emergency mouth-to-mouth resuscitation, mouth-pieces, resuscitation bags, or other ventilation devices should be available for use in areas where the need for resuscitation is predictable.
20. Health care workers who have exudative lesions or weeping dermatitis should refrain from all direct patient care and from handling patient-care equipment until the condition is resolved.

Informed Consent Form
for
The Rules and Regulations in Practice Laboratory

1. All materials and clothes other than those needed for the laboratory are to be kept away from the work area.
2. A lab coat or other protective clothing must be worn during lab. The lab clothing is not to be worn outside of the laboratory.
3. Clean the lab table before and after lab with the disinfectant solution provided.
4. Wash hands before leaving lab.
5. Any item contaminated with bacteria or body fluids must be disposed of properly. Disposable items are to be placed in the BIOHAZARD container. Reusable items are to be placed in the designated area for autoclaving prior to cleaning. Sharps are to be disposed of in the appropriate container
6. Reusable items should have all tape and marks removed by the student before being autoclaved.
7. Because organisms used in this class are potentially pathogenic, aseptic technique must be observed at all times. No eating, drinking, application of cosmetics or smoking is allowed. Mouth pipetting is not allowed.
8. Cuts and scratches must be covered with Band-Aids. Disposable gloves will be provided on request.
9. Long hair should be tied back while in lab.
10. All accidents, cuts, and any damaged glassware or equipment should be reported to the lab instructor immediately.
11. Sterilization techniques will involve the use of Bacticinerators that are fire and burn hazards.
12. Micro-incinerators reach an internal temperature of 850 ^{0}C. Keep all combustibles away from the micro-incinerators. Do not leave inoculating loops or needles propped in the micro-incinerators.
13. Microscopes and other instruments are to be cared for as directed by the instructor.
14. It is the responsibility of the student to know the location and use of all safety equipment in the lab (eyewash, fire extinguisher, etc.).
15. Cultures may not be removed from the lab. Visitors are not allowed in the lab.
16. Doors and windows are to be kept closed at all times.
17. For the best lab experience, read labs before coming to class. Make notes as necessary. Wait for a laboratory introduction by the instructor before starting work.

I have read and understand the above rules and agree to follow them.

Signed_____ Date_____
Name (Please print)_____



Reference: Modified from http://faculty.ccc.edu/tr-infectioncontrol/chain.htm

Interventional opportunities to break the chain of infection by various intervention modalities such as *vaccination, sterilization, disinfection, decontamination, antisepsis, hygiene, sanitation, biosafety precautions, prohylaxis, treatment*, etc.

First Aid Procedures
after
Accidental Exposure to Infectious Material

• Accidental sharps injury

What should you know?

- *A significant exposure risk is present in any accidental sharps injury, even if no blood is visible and the skin does not appear to be broken.*

What should you do?

- Flush the area well in clean running water and wash thoroughly with soap.
- Cover with a dressing if necessary.
- Report the incident to a supervisor or the physician-in-charge immediately.
- Corrective action is required if a procedural cause of the accident is identified.

• Accidental contact with infectious material

What should you know?

- *This includes any unprotected contact between potentially infectious material and broken skin, the mouth, nose or eye.*

What should you do?

- Flush the area with soap and clean water. Use water or sterile saline alone for splashes to the eye or mouth.
- Report the incident to a supervisor or the physician-in-charge immediately.
- Corrective action is required if a procedural cause of the accident is identified.

• Immediate actions after accidental exposure

What should you know?

- Certain procedures must be followed after exposure to potentially infectious material.
- Patients may be infected with other pathogens unrelated to the outbreak investigation, for example hepatitis B virus or HIV.

What should you do?

- A baseline blood specimen should be collected immediately from the exposed health care worker and, if feasible, from the source.
- Prophylactic measures should be initiated immediately for all of the workers exposed.
- If there is a risk for outbreak, procedures for possible treatment and for the longer term follow-up of exposed health care workers should be established.
- Corrective action is required if a procedural cause of the accident is identified.

Decontamination of surfaces

- Wear an apron, heavy-duty gloves, and other barrier protection if needed.
- Wipe clean with an absorbent material.
- Disinfect surface by wiping clean with the disinfectant given.
- Then discard all absorbent material in heavy duty “hazardous waste” with a red bag.

Decontamination of blood or body fluid spills

- Learn the location and how to use the equipment in case of an accidental spill.
- Put an absorbent tissue with a disinfectant on the spillage or splash and left for at least 30 minutes.

Hand Hygiene

Hand washing (plain soap and water, paper towel)
Hand rubbing (alcohol, no water, no towel)
Surgical Hand Preparation

What should you know?

The skin is the biggest organ in the body and approximately 1.5 m^2.
Normal skin has aerobic microorganisms in flora:

- Different number of organism in different region of the skin
  - Scalp $1x10^6$
  - Axillary $5x10^5$
  - Abdomen $4x10^4$
  - Forearm $1x10^4$ CFU/cm^2
- Bacterial counts on healthcare workers' (HCW) hands range from 10^4 to 10^6/cm^2.

Skin Flora

- **Resident microorganisms;**
  - attached to deeper layers of the skin more resistant to removal less likely to be associated with healthcare associated infections (HAIs).

- **Transient microorganisms;**
  - acquired by direct contact with patients or contaminated environmental surfaces
  - colonize the superficial layers of skin
  - amenable to removal
  - frequently associated with HAIs

Personnel contaminate hands by performing "clean procedures" such as;

- Lifting patients
- Taking the patient's pulse
- Taking blood pressure
- Taking oral temperature
- Touching the patient's hand, shoulder, or groin
- Nurses contaminate hands with 100-1000 CFU during clean activities

What should you do?

Perform hand hygiene:

- Before;
  - Patient contact
  - Donning gloves when inserting a central venous catheter (CVC)
  - Before handling an invasive device for patient care, regardless of whether or not gloves are used

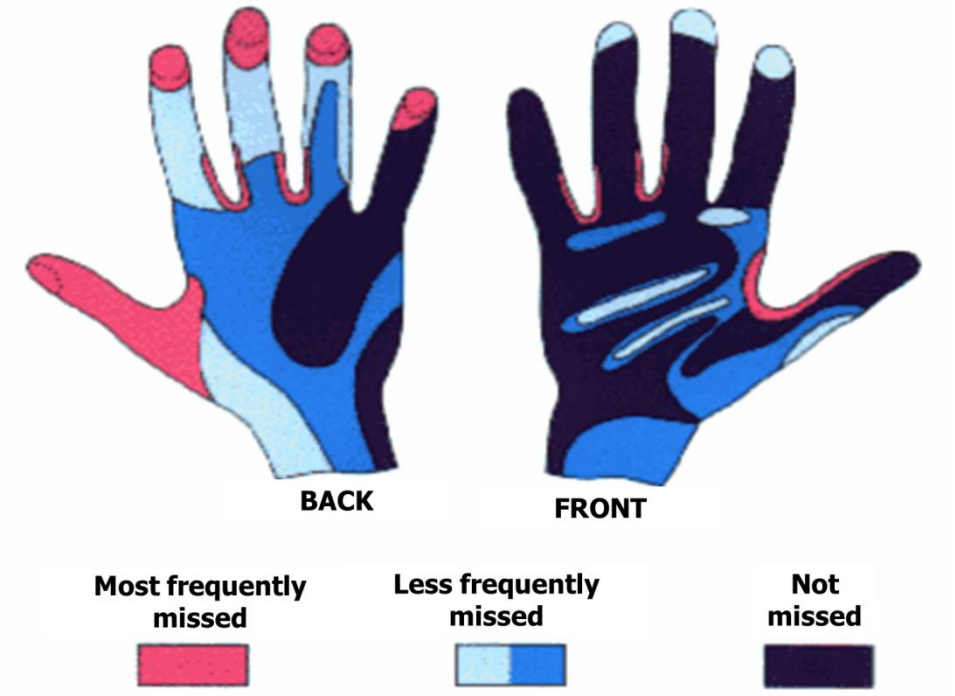
    - Urinary catheter, peripheral vascular catheter, or other invasive devices
- After;
    - Direct contact with patients skin
    - If moving from a contaminated body site to a clean body site during patient care
    - Contact with body fluids or excretions, mucous membranes, non-intact skin, or wound dressings
    - Contact with inanimate objects (including medical equipment) in the immediate vicinity of the patient
    - Removing gloves

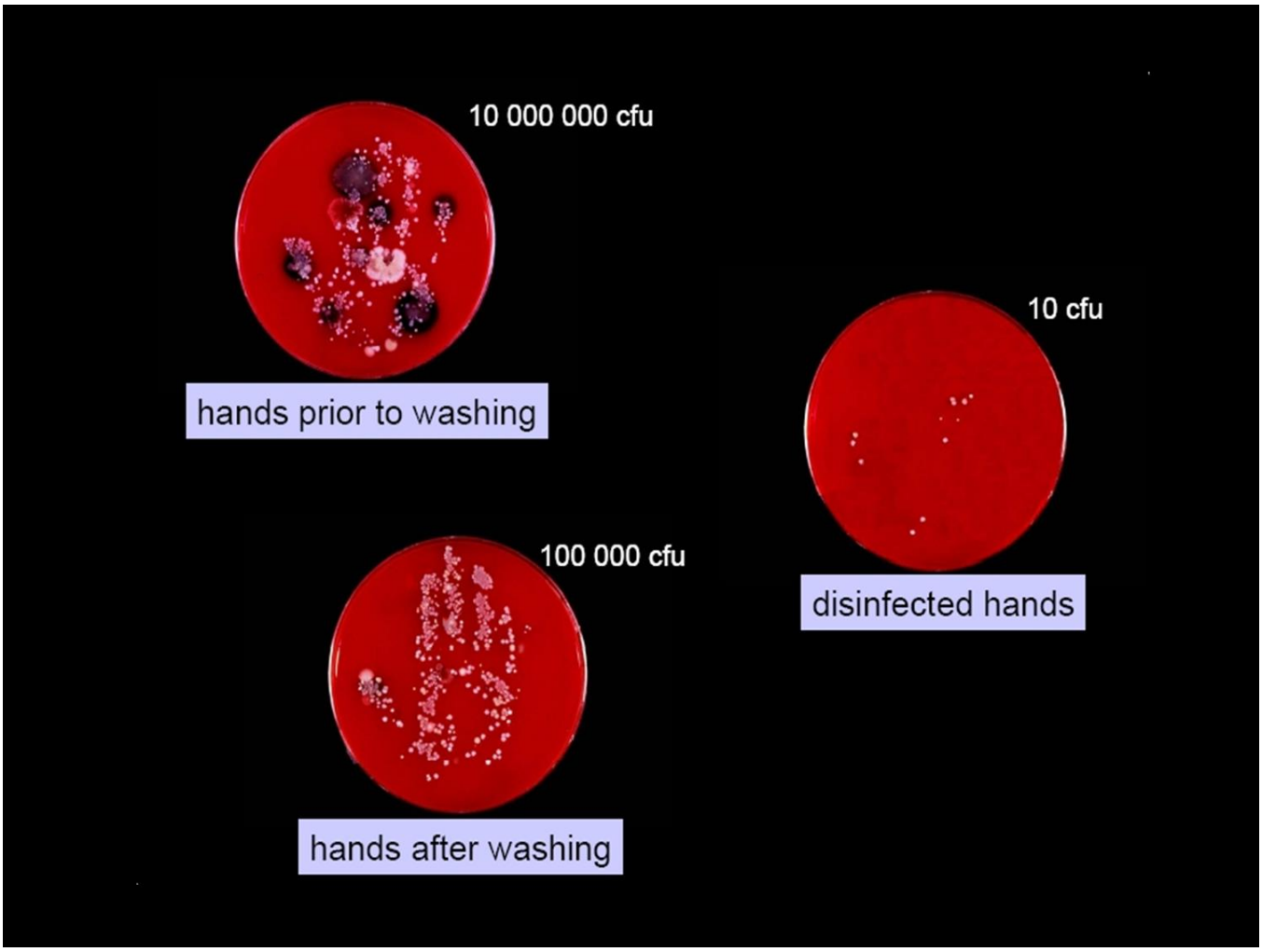
Wash hands with soap and water:

- When visibly dirty or contaminated with proteinaceous material
- Visibly soiled with blood or other body fluids
- If exposure to potential spore-forming organisms is strongly suspected or proven
- After the restroom

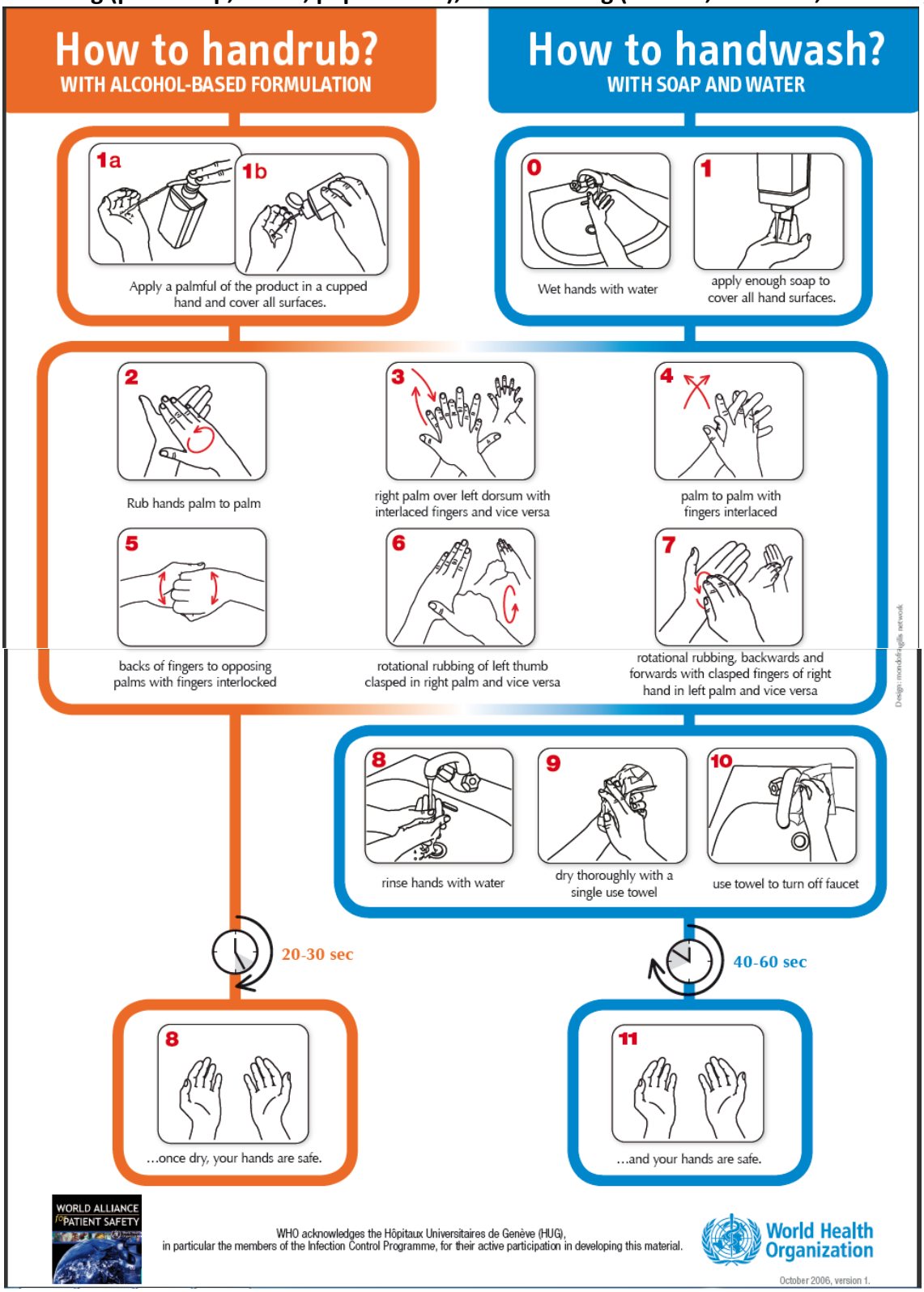
Use an alcohol-based hand rub for routine hand antisepsis:

- In all other clinical situations
- If hands are not visibly soiled
- Alternatively, wash hands with soap and water




10 000 000 cfu
hands prior to washing
10 cfu
disinfected hands
100 000 cfu
hands after washing

Hand washing (plain soap, water, paper towel), Hand rubbing (alcohol, no water, no towel)



Reference: *WHO Global Patient Safety Challenge, 2006, WHO, Geneva. (http://www.who.int/gpsc/tools/GPSC-HandRub-Wash.pdf)*

Surgical Hand Preparation

Figure I.13.1
Surgical hand preparation technique with an alcohol-based handrub formulation

The handrubbing technique for surgical hand preparation must be performed on perfectly clean, dry hands.
On arrival in the operating theatre and after having donned theatre clothing (cap/hat/bonnet and mask), hands must be washed with soap and water.
After the operation when removing gloves, hands must be rubbed with an alcohol-based formulation or washed with soap and water if any residual talc or biological fluids are present (e.g. the glove is punctured).

Surgical procedures may be carried out one after the other without the need for handwashing, provided that the handrubbing technique for surgical hand preparation is followed (Images 1 to 17).

1
Put approximately 5ml (3 doses) of alcohol-based handrub in the palm of your left hand, using the elbow of your other arm to operate the dispenser

2
Dip the fingertips of your right hand in the handrub to decontaminate under the nails (5 seconds)

3
Images 3–7: Smear the handrub on the right forearm up to the elbow. Ensure that the whole skin area is covered by using circular movements around the forearm until the handrub has fully evaporated (10-15 seconds)

4
See legend for Image 3

5
See legend for Image 3

6
See legend for Image 3

7
See legend for Image 3

8
Put approximately 5ml (3 doses) of alcohol-based handrub in the palm of your right hand, using the elbow of your other arm to operate the dispenser

9
Dip the fingertips of your left hand in the handrub to decontaminate under the nails (5 seconds)

Reference: *WHO Guidelines on Hand Hygiene in Health Care, 2009, WHO, Geneva.*

Figure I.13.1
Surgical hand preparation technique with an alcohol-based handrub formulation (Cont.)

10
Smear the handrub on the left forearm up to the elbow. Ensure that the whole skin area is covered by using circular movements around the forearm until the handrub has fully evaporated (10-15 seconds)

11
Put approximately 5ml (3 doses) of alcohol-based handrub in the palm of your left hand, using the elbow of your other arm to operate the distributor. Rub both hands at the same time up to the wrists, and ensure that all the steps represented in Images 12-17 are followed (20-30 seconds)

12
Cover the whole surface of the hands up to the wrist with alcohol-based handrub, rubbing palm against palm with a rotating movement

13
Rub the back of the left hand, including the wrist, moving the right palm back and forth, and vice-versa

14
Rub palm against palm back and forth with fingers interlinked

15
Rub the back of the fingers by holding them in the palm of the other hand with a sideways back and forth movement

16
Rub the thumb of the left hand by rotating it in the clasped palm of the right hand and vice versa

17
When the hands are dry, sterile surgical clothing and gloves can be donned

Repeat the above-illustrated sequence (average duration, 60 sec) according to the number of times corresponding to the total duration recommended by the manufacturer for surgical hand preparation with an alcohol-based handrub.

Reference: *WHO Guidelines on Hand Hygiene in Health Care, 2009, WHO, Geneva.*

2.1. Collection, Storage and Transport of Specimens

Laboratory safety

WHO Risk Groups for Infectious Disease Agents

Table 1. ***Classification of infective microorganisms by risk group***

Risk Group 1 *(no or low individual and community risk)*
A microorganism that is unlikely to cause human or animal disease.

Risk Group 2 *(moderate individual risk, low community risk)*
A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.

Risk Group 3 *(high individual risk, low community risk)*
A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

Risk Group 4 *(high individual and community risk)*
A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

WHO Risk Groups (RG) and Laboratory Biosafety Levels (BSL)

Table 2. ***Relation of risk groups to biosafety levels, practices and equipment***

RISK GROUP	BIOSAFETY LEVEL	LABORATORY TYPE	LABORATORY PRACTICES	SAFETY EQUIPMENT
1	Basic – Biosafety Level 1	Basic teaching, research	GMT	None; open bench work
2	Basic – Biosafety Level 2	Primary health services; diagnostic services, research	GMT plus protective clothing, biohazard sign	Open bench plus BSC for potential aerosols
3	Containment – Biosafety Level 3	Special diagnostic services, research	As Level 2 plus special clothing, controlled access, directional airflow	BSC and/or other primary devices for all activities
4	Maximum containment – Biosafety Level 4	Dangerous pathogen units	As Level 3 plus airlock entry, shower exit, special waste disposal	Class III BSC, or positive pressure suits in conjunction with Class II BSCs, double-ended autoclave (through the wall), filtered air

BSC, biological safety cabinet; GMT, good microbiological techniques (see Part IV of this manual)

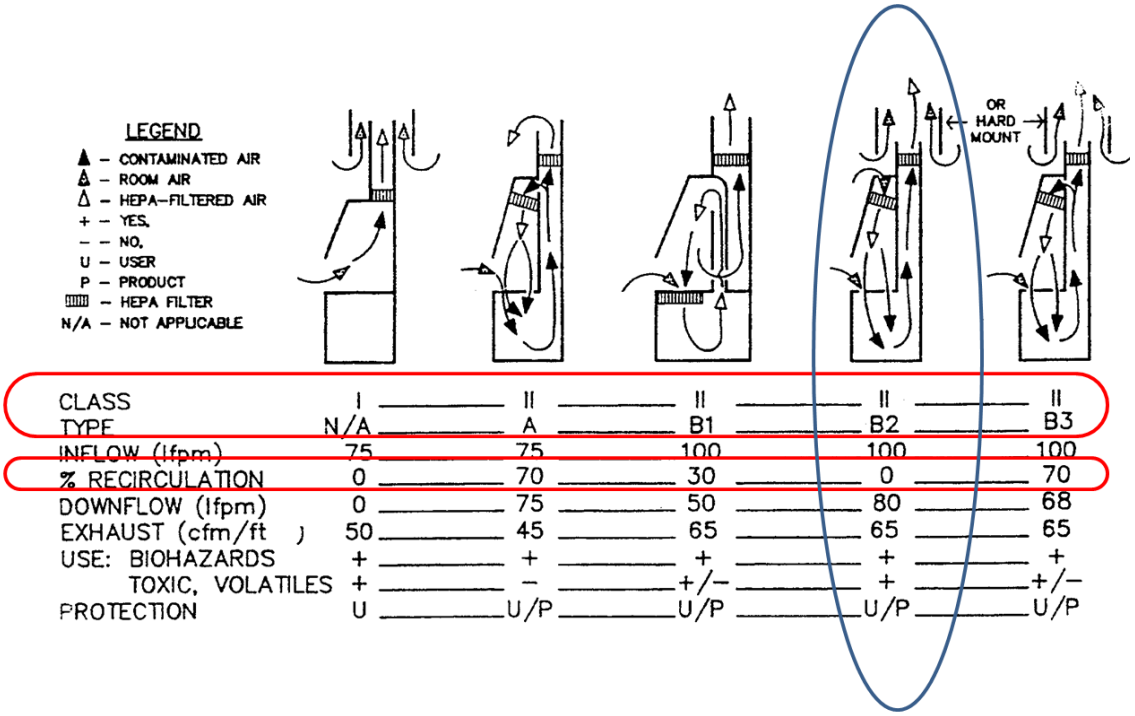
Reference: *Laboratory Biosafety Manual, WHO, Geneva, 2004. (http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf)*

Laboratory Biosafety Levels (BSL)

Biological agents by risk *versus* biosafety levels					
BSL	**WHO Risk Group**	**Exemplary Agents**	**Biosafety Practices**	**Biosafety Equipment (Primary Barriers)**	**Physical Facilities (Secondary Barriers)**
1	1 *(Agents that are not likely to be a cause of infectious disease in healthy human)*	Normal microbiota of human	Standard microbiological practices	Not required	Open bench top sink required
2	2 *(Agents that may be associated with human disease through exposure by various routes; e.g. percutaneous injury, ingestion, or mucous membrane exposure)*	*Enterobacteriaceae*, *Candida* spp., *Mycobacteria*, *Human Herpes Viruses*	BSL-1 plus: • Limited access • Biohazard warning signs • "Sharps" precautions • Biosafety manual defining waste management and medical surveillance practices	Class I or II BSCs and other physical containment devices used for all manipulations of agents that may cause splashes or aerosols of infectious materials. PPEs *(e.g. laboratory coats, gloves, face protection shield, etc.)*	BSL-1 plus: • Autoclave available
3	3 *(Agents that may cause disease with serious or lethal consequence through aerosol transmission)*	*Mycobacterium tuberculosis*, *Franciscella tularensis*, *Brucella* spp. (if colony will be manipulated), West Nile virus	BSL-2 practices plus: • Controlled access • Decontamination of all waste • Decontamination of laboratory clothing before laundering • Baseline serum	Preferably BSC Class II Type B2	BSL-2 plus: • Physical separation from access corridors • Self-closing. double-door access • Exhausted air not re-circulated • Negative airflow into laboratory
4	4 *(Agents which pose high risk of life threatening diseases or aerosol-transmitted laboratory infections. Or agents with unknown risk of transmission)*	*Arenaviruses (that produce hemorrhagic fever)* *Filoviruses (that produce hemorrhagic fever; Marburg, Ebola)*	BSL-3 practices plus: • Clothing change on entering • Shower on exit • All materials decontaminated on exit from facility	All procedures conducted in Class III BSCs or in BCS CII TB2 *(in combination with full body, air-supplied positive pressure personnel suit)*	BSL.-3 plus: • Separate building or isolated zone • Dedicated supply and exhaust, vacuum, and decontaminated systems • Further requirements.

Reference: *Modified from Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Biosafety Cabinets (BSC)



Reference: *NCCLS. Laboratory Design; Approved Guideline. NCCLS document GP18-A, NCCLS, Wayne, Pennsylvania, 1998.*

Laboratory Design: Biosafety Level 2

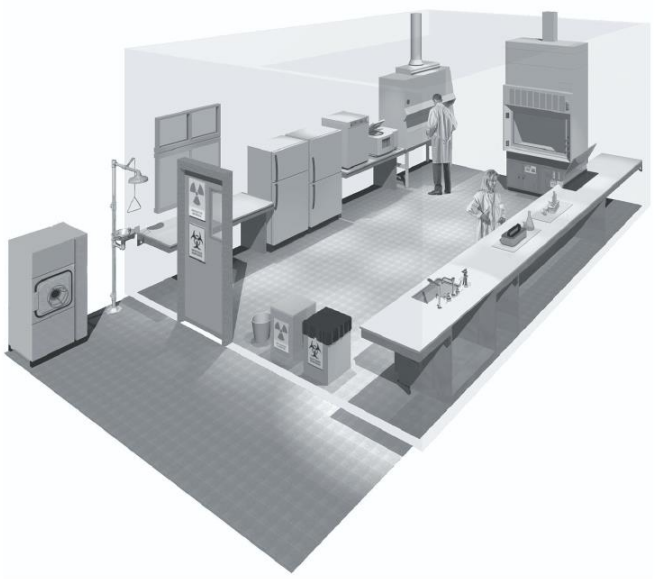


Figure 3. ***A typical Biosafety Level 2 laboratory*** *(graphics kindly provided by CUH2A, Princeton, NJ, USA). Procedures likely to generate aerosols are performed within a biological safety cabinet. Doors are kept closed and are posted with appropriate hazard signs. Potentially contaminated wastes are separated from the general waste stream.*

Reference: *Laboratory Biosafety Manual, WHO, Geneva, 2004. (http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf).*

Laboratory Design: Biosafety Level 3

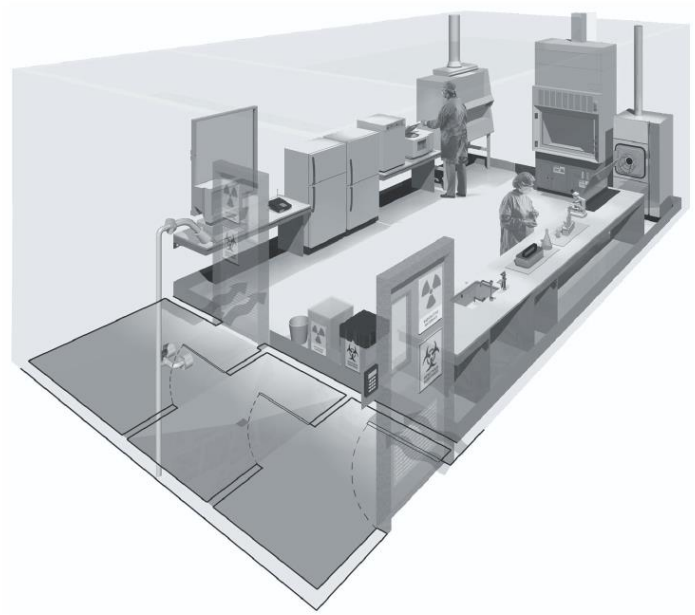


Figure 4. ***A typical Biosafety Level 3 laboratory***
(graphics kindly provided by CUH2A, Princeton, NJ, USA). The laboratory is separated from general traffic flow and accessed through an anteroom (double door entry or basic laboratory – Biosafety Level 2) or an airlock. An autoclave is available within the facility for decontamination of wastes prior to disposal. A sink with hands-free operation is available. Inward directional airflow is established and all work with infectious materials is conducted within a biological safety cabinet.

Reference: *Laboratory Biosafety Manual, WHO, Geneva, 2004. (http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf).*

Specimen Requisition for Diagnostic Tests

A complete requisition should include the following:

- The patient's name
- Hospital number
- Age or date of birth
- Sex
- Collection date and time
- Ordering physician
- Specimen type and collection technique
- Preliminary diagnosis
- Immunization history
- Current antimicrobial therapy

Specimen Rejection Criteria (General)

- Missing or inadequate identification
- Information mismatch between the label and requisition.
- Inappropriate transport or storage conditions (container, medium, atmospheric condition, temperature, time, leakage, preservation, etc.)
- Insufficient quantity for testing.
- Unknown time delay or more than 2 hours transport time after past post-collection without preservation.
- Specimen collected in a fixative (e.g. formalin; kills all microorganisms present)
- Collection of specimen for anaerobic culture from a site with anaerobic flora (e.g. mouth, vagina)
- Dried up specimen
- Inadvisable specimen types or specimens collected through imprudent collection techniques.
- Suspected contamination
- Haemolysed blood sample

References:

Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.

Bailey & Scott's Diagnostic Microbiology. Forbes B.A., Sahm D.F., Weissfeld A.S. 12th ed. Mosby Elsevier, Missouri.

Specimen Types, Collection Techniques, Storage and Transport Requirements

- Microbiologist is responsible for providing the clinicians with a manual or instruction card listing optimal procedures for specimen collection, temporary storage and transport;
    - Basic information should include:
        - Safety requirements
        - Limitations (analytical, diagnostic)
        - Advantages (supported medical decision types)
        - Disadvantages (risks, complications)
        - Patient preparation
        - Illuminated consent
        - Selection of appropriate anatomic site
        - Selection of appropriate specimen type
        - Selection of appropriate collection technique
        - Quantity
        - Replica limits
        - Labeling requirements
        - Temporary storage and transport conditions
        - Patient care
    - Additional information may include:
        - Reminders such as;
            - Specimens should be collected preferably;
                - during the acute/early phase of the infection (within 2 or 3 days for viral infections)
                - before antimicrobials are administered
            - If available, tissue or needle aspirates preferred instead of swab specimens
        - Available test menu, reference ranges, test methods/techniques, medical decision limits, panic values, turn-around-times.

- **The laboratory diagnosis of an infectious disease begins with the collection of a clinical specimen for examination or processing;**
  - the right one
  - collected at the right time
  - transported in the right way
  - to the right laboratory
  - Proper collection of an appropriate clinical specimen is the first step in obtaining **an accurate laboratory diagnosis** of an infectious disease.
- **Guidelines for the collection and transportation of specimens:**
  - collection of the specimen before the administration of antimicrobial agents
  - prevention of contamination of the specimen with externally present organisms or normal flora of the body
  - apply strict aseptic techniques throughout the procedure
  - wash hands before and after the collection
  - collect the specimen at the appropriate phase of disease
  - make certain that the specimen is representative of the infectious process (e.g. sputum is the specimen for pneumonia and not saliva) and is adequate in quantity for the desired tests to be performed
  - collect or place the specimen aseptically in a sterile and/or appropriate container
  - ensure that the outside of the specimen container is clean and uncontaminated
  - close the container tightly so that its contents do not leak during transportation
  - label and date the container appropriately and complete the requisition form
  - arrange for immediate transportation of the specimen to the laboratory

References:

Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.

Bailey & Scott's Diagnostic Microbiology. Forbes B.A., Sahm D.F., Weissfeld A.S. 12th ed. Mosby Elsevier, Missouri.

Selection of common clinical specimens for bacterial culture[a]

Anatomic site	Clinical specimen(s)	
	Appropriate	Inappropriate
Lower respiratory tract	Freshly expectorated mucus and inflammatory cells (pus), sputum	Saliva, oropharyngeal secretions, sinus drainage from nasopharynx
Sinus	Secretions collected by direct sinus aspiration, or washes, curettage, and biopsy material collected during endoscopy	Nasal or nasopharyngeal swab, nasopharyngeal secretions, sputum, and saliva
Urinary tract	Midstream urine, urine collected by "straight" catheterization, urine collected by suprapubic aspiration, urine collected during cystoscopy or other surgical procedure	Urine from Foley catheter collection bag, "bagged" urine from infants
Superficial wound	Aspirations of pus or local irrigation fluid (nonbacteriostatic saline), swab of purulence originating from beneath the dermis	Swab of surface material or specimen contaminated with surface material, irrigation with saline containing preservative
Deep wound	Purulence, necrosis, or tissue from deep subcutaneous site	Specimen contaminated with surface material
Gastrointestinal tract	Freshly passed stool, washes, or feces collected during endoscopy; rectal swab (in selected cases)	Rectal swab, specimen for bacterial culture if diarrhea developed after patient was in hospital for >3 days
Venous blood	Two or three blood specimens collected from separate venipunctures, before initiation of antibiotics, each containing approx 20 ml of blood for patients >90 lb (see Table 7 for pediatric volumes); antisepsis *with iodine-containing compound* or chlorhexidine	Clotted blood; one or more than three blood specimens collected within a 24-h period; vol of blood <20 ml per culture (i.e., per venipuncture); antisepsis with alcohol only (adults)

Reference: *Murray P.R. (Ed.), Manual of Clinical Microbiology, ASM Press, Washington, 2007.*

Suitability of various specimens for anaerobic culture

Acceptable material (method of collection)	Unacceptable material
Aspirate (by needle and syringe)	Bronchoalveolar lavage washing
Bartholin's gland inflammation/secretions	Cervical secretions
Blood (venipuncture)	Endotracheal secretions (aspirate)
Bone marrow (aspirate)	Lochia secretions
Bronchoscopic secretions (protected specimen brush)	Nasopharyngeal swab
Culdocentesis fluid (aspirate)	Perineal swab
Fallopian tube fluid or tissue (aspirate/biopsy)	Prostatic or seminal fluid
IUD, for *Actinomyces* spp.	Sinus washings or swabs
Nasal sinus (aspirate)	Sputum (expectorated or induced)
Placenta tissue (via cesarean delivery)	Stool or rectal swab samples
Stool, for *C. difficile*	Tracheostomy secretions
Surgical site (aspirate, tissue)	Urethral secretions
Transtracheal aspirate	Urine (voided or from catheter)
Urine (suprapubic aspirate)	Vaginal or vulvar secretions (swab)

Reference: *Murray P.R. (Ed.), Manual of Clinical Microbiology, ASM Press, Washington, 2007.*

TABLE 3 Bacteriology collection, transport, and storage guidelines[a]

Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Abs						
General	Remove surface exudate by wiping with sterile saline or 70% alcohol.					Tissue or aspirate is always superior to a swab specimen. If swabs must be used (aerobic culture only), collect two, one for culture and one for Gram staining. Preserve swab material by placing in Stuart's or Amies medium.
Open	Aspirate if possible or pass a swab deep into the lesion to firmly sample the lesion's "fresh border."	Swab transport system	≤2 h, RT	≤24 h, RT	1/day/source	Samples of the base of the lesion and abscess wall are most productive.
Closed	Aspirate abscess material with needle and syringe. Aseptically transfer *all* material into anaerobic transport device.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day/source	Contamination with surface material will introduce colonizing bacteria not involved in the infectious process. Do not use syringe for transport.
Bite wound	See Abscess					Do not culture animal bite wounds ≤12 h old (agents are usually not recovered) unless signs of infection are present.
Blood (15, 160)	Disinfect culture bottle; apply 70% isopropyl alcohol or phenolic disinfectant to rubber stoppers and wait 1 min. Palpate vein before disinfection of venipuncture site. Disinfection of venipuncture site: 1. Cleanse site with 70% alcohol. 2. Swab concentrically, starting at the center, with tincture of iodine or chlorhexidine. 3. Allow the disinfectant to dry. 4. *Do not palpate vein at this point without sterile glove.*	Blood culture bottles for bacteria; adult, 20 ml/set (higher vol most productive); infant and child, 1–20 ml/set depending on wt of patient (see Table 7)	≤2 h, RT	≤2 h, RT or per instructions	3 sets in 24 h	Acute febrile episode: 2 sets[c] from separate sites, all within 10 min (before antimicrobials) Nonacute disease, antimicrobials will not be started or changed immediately: 2 or 3 sets from separate sites, all within 24 h at intervals no closer than 3 h (before antimicrobials) Endocarditis, acute: 3 sets from 3 separate sites, within 1–2 h, before antimicrobials if possible Fever of unknown origin: 2 or 3 sets from separate sites ≥1 h apart during 24-h period. If negative at 24–48 h, obtain 2 3 more sets. e data indicate that an additional robic or fungal bottle is more productive than the anaerobic bottle.

Reference: *Murray P.R. (Ed.), Manual of Clinical Microbiology, ASM Press, Washington, 2007.*

	2. Aspirate the area of maximum inflammation (commonly the center rather than the leading edge) with a needle and syringe. Irrigation with a small amount of sterile saline may be necessary. 3. Aspirate saline into syringe and expel into sterile screw-cap tube.					
CSF	1. Disinfect site with iodine preparation. 2. Insert a needle with stylet at L3-L4, L4-L5, or L5-S1 interspace. 3. Upon reaching the subarachnoid space, remove the stylet and collect 1–2 ml of fluid into each of 3 leakproof tubes.	Sterile screw-cap tubes Minimum amt required: bacteria, ≥1 ml	Bacteria: never refrigerate; ≤15 min, RT	≤24 h, RT	None	Obtain blood for culture also. If only 1 tube of CSF is collected, it should be submitted to microbiology first; otherwise submit tube 2 to microbiology. Aspirate of brain abscess or a biopsy sample may be necessary to detect anaerobic bacteria or parasites.
Decubitus ulcer (17)	A swab is not the specimen of choice (see comments). 1. Cleanse surface with sterile saline. 2. If a biopsy sample is not available, aspirate inflammatory material from the base of the ulcer.	Sterile tube/container (aerobic) or anaerobic system (for tissue)	≤2 h, RT	≤24 h, RT	1/day/source	Since a swab specimen of a decubitus ulcer provides no clinical information, it should not be submitted. A tissue biopsy sample or needle aspirate is the specimen of choice.
Dental culture: gingival, periodontal, periapical, Vincent's stomatitis	1. Carefully cleanse gingival margin and supragingival tooth surface to remove saliva, debris, and plaque. 2. Using a periodontal scaler, carefully remove subgingival lesion material and transfer it to an anaerobic transport system. 3. Prepare smear for staining with specimen collected in the same fashion.	Anaerobic transport system	≤2 h, RT	≤24 h, RT	1/day	Periodontal lesions should be processed only by laboratories equipped to provide specialized techniques for the detection and enumeration of recognized pathogens.

Reference: *Murray P.R. (Ed.), Manual of Clinical Microbiology, ASM Press, Washington, 2007.*

Respiratory, upper Oral	1. Remove oral secretions and debris from the surface of the lesion with a swab. Discard this swab. 2. Using a second swab, vigorously sample the lesion, avoiding any areas of normal tissue.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Discourage sampling of superficial tissue for bacterial evaluation. Tissue biopsy specimens or needle aspirates are the specimens of choice.
Nasal	1. Insert a swab, premoistened with sterile saline, approx 1–2 cm into the nares. 2. Rotate the swab against the nasal mucosa.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Anterior nose cultures are reserved for identifying staphylococcal carriers or for nasal lesions.
Nasopharynx (25)	1. Gently insert a small swab (e.g., calcium alginate) into the posterior nasopharynx via the nose. 2. Rotate swab slowly for 5 s to absorb secretions.	Direct medium inoculation at bedside or examination table, swab transport	Plates: ≤15 min, RT Swabs: ≤2 h, RT	≤24 h, RT	1/day	
Throat or pharynx	1. Depress tongue with a tongue depressor. 2. Sample the posterior pharynx, tonsils, and inflamed areas with a sterile swab.	Swab transport (dry swab with or without silica gel is good for *S. pyogenes* and *C. diphtheriae*)	≤2 h, RT	≤24 h, RT	1/day	Throat swab cultures are contraindicated for patients with epiglottitis. Swabs for *N. gonorrhoeae* should be placed in charcoal-containing transport medium and plated ≤12 h after collection. JEMBEC, Bio-Bags, and the GonoPak are better for transport at RT.
Tissue	Collected during surgery or cutaneous biopsy procedure	Anaerobic transport system or sterile, screw-cap container. Add several drops of sterile saline to keep small pieces of tissue moist.	≤15 min, RT	≤24 h, RT	None	Always submit as much tissue as possible. If excess tissue is available, save a portion of surgical tissue at −70°C in case further studies are needed. Never submit a fb that has been rubbed over the face of a tissue. For quantitative study, ample of 1 cm³ is appropriate.

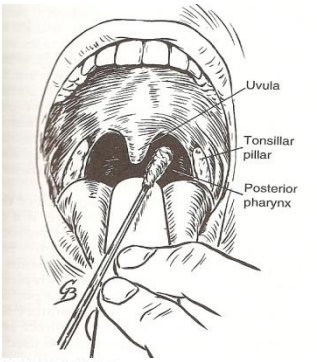
Reference: *Murray P.R. (Ed.), Manual of Clinical Microbiology, ASM Press, Washington, 2007.*

Collection of a throat swab specimen

- **Specimen collection:**

a. Place gentle pressure on the tongue with a tongue depressor.
b. Extend one or two sterile swabs (one for the antigen test and one for culture if necessary) between the tonsillar pillars and behind the uvula, avoiding the tongue, buccal mucosa, and uvula.
c. Sweep the swabs back and forth across the posterior pharynx, tonsillar areas, and any inflamed or exudative areas.
d. Do not obtain specimens if the epiglottis is inflamed, since sampling may trigger life-threatening respiratory obstruction.

The patient is asked to open the widely and phonate an "ah." The tongue is gently depressed and with a tongue blade and a swab is guided over the tongue into the posterior pharynx. The mucosa behind and between the tonsillar pillars is swabbed with back-and-forth sweeping motion.



Specimen type (reference)	Collection guidelines	Transport device and/or minimum vol	Transport[b] time and temp	Storage time and temp	Replica limits	Comments
Throat or pharynx	1. Depress tongue with a tongue depressor. 2. Sample the posterior pharynx, tonsils, and inflamed areas with a sterile swab.	Swab transport (dry swab with or without silica gel is good for *S. pyogenes* and *C. diphtheriae*)	≤2 h, RT	≤24 h, RT	1/day	Throat swab cultures are contraindicated for patients with epiglottitis. Swabs for *N. gonorrhoeae* should be placed in charcoal-containing transport medium and plated ≤12 h after collection. JEMBEC, Bio-Bags, and the GonoPak are better for transport at RT.

References:

Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.

Murray P.R. (Ed.), Manual of Clinical Microbiology, ASM Press, Washington, 2007.

3.1. Microscopy Methods in Diagnostic Microbiology

The Study of Cell Morphology

Wet-mount Technique

Observation of living bacteria (prokaryotic) and yeast (eukaryotic) cells by wet-mount technique

What should you know?

The direct examination of living microorganisms and cells can be quite useful in determining their natural sizes and shapes (without distortion that can occur with drying and fixation), observing motility, and detecting the organisms' reactions to various chemicals.

There are two common methods for examining living microorganisms with a bright-field microscope:

- **the wet-mount technique**
- the hanging-drop technique

With wet-mount method, a drop of the sample is place on a clean slide and a cover slip is added. It is quick and easy. But fluid drifting out and evaporation are the main problems for this technique. Therefore after the specimen is prepared it is advised to do the microscopic observations as soon as possible.

What should you do?

Materials and Methods:

1. Overnight (16-18 hours of incubation) culture plates of *Escherichia coli* (shape: bacillus), *Staphylococcus aureus* (shape: coccus), and *Candida albicans* (yeast: budding coccus shaped).
2. Microscope slides and cover slips.
3. Inoculating loops and micro-incinerators.
4. Lens paper, immersion oil, lab. marker, and microscope
5. Container for culture disposal
6. Disinfectant solution in beakers
7. Saline solution

1. Prepare seperate wet-mounts from each of the culture plates containing *Escherichia coli, Staphylococcus aureus, Candida albicans .*
    a. Place one drop (40 µL) of saline solution to the center position of the glass slide.
    b. Flame the inoculating loop to redness.
    c. Remove the plug from the culture, and briefly flame the tip of the tube.
    d. Remove a loopful of the culture isolate (pure colony) and emulsify the colony in the saline solution on the glass slide.
    e. Reflame the tip of the culture tube and replace the plug.
    f. Reflame the inoculating loop before putting it down.
    g. Gently place a cover slip on the droplet.

2. Examine under low power (10x10 magnification power) and high power fields (10X40 magnification power), respectively.

3. Sketch a representative view of the samples on your **'Results'** section.

4. Discard the slides in the disinfectant container as indicated and wipe the objectives.

5. On your **'Discussion**' section write and discuss:

    a. *Which sample was easy to observe?*
    b. *From which objective did you observe your samples most easily and why?*
    c. *Which type of cells were most easily evaluated using this technique with which objective*?

LABORATORY REPORT

DATE:

TITLE:

INTRODUCTION (Purpose of the study):

MATERIALS AND METHODS:

RESULTS:

DISCUSSION/CONCLUSION:

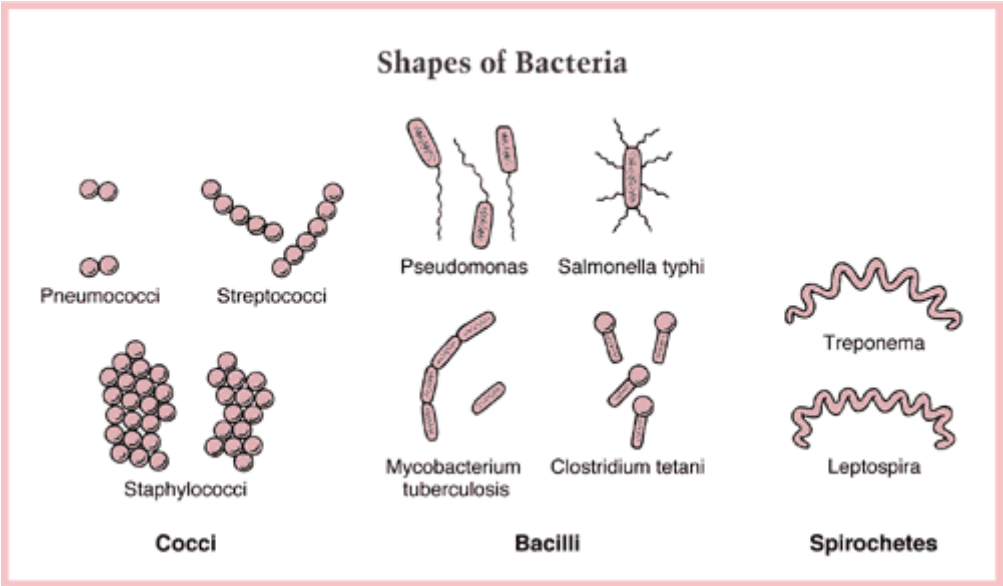
Gram Stain
(Smear Preparation, Fixation, Staining, Microscopic Examination)

What should you know?

The purpose of this laboratory study is to show you how to prepare (i.e. smear), fix, and stain a microbial culture (i.e. bacterial isolate in the form of a pure colony on solid culture medium) on a glass microscope slide, so that you can determine the morphology of cells in a culture or in a patient specimen.

Generally, the shapes and sizes of microorganisms are genetically determined, and most bacteria have very rigid cell walls, although changes in environment may have some small effect on their sizes and shapes. Therefore, the shapes and sizes of microorganisms are important characteristics that you can use for their identification.

Basic Bacterial Shapes
(Microscopic morphology of cells)



- **Gram stain** :
  - **is a rapid (less than 10 minutes), powerful, easy test that allows clinicians to distinguish between the two major classes of bacteria,**
  - **develop an initial diagnosis, and initiate therapy based on inherent differences in the bacteria**
    - bacteria are heat fixed or otherwise dried onto a slide,
    - stained with crystal violet , stain that is precipitated with iodine,
    - and then the unbound and excess stain is removed by washing with the acetone-based decolorizer and water.
    - a red counterstain, safranin, is added to stain any decolorized cells.
- **Gram-positive bacteria**;
  - turns into **purple**, the stain gets trapped in a thick, cross-linked, mesh-like structure, the peptidoglycan layer, which surrounds the cell
    - a mnemonic device that may help is "P-PURPLE-POSITIVE."
- **Gram-negative bacteria**;
  - have a thin peptidoglycan layer that does not retain the crystal violet stain, so the cells must be counterstained with safranin and turned **red.**
- *Due to degradation of the peptidoglycan;*
  - ***Gram stain is not a dependable test for bacteria that are;***
    - ***starved (e.g., old or stationary phase cultures) or***
    - ***treated with antibiotics.***
- **Bacteria that cannot be classified by Gram stain include;**
  - **mycobacteria**, which have a waxy outer shell and are distinguished with **the acid-fast stain**, and
  - **mycoplasmas**, which have **no peptidoglycan**.

The Gram (i.e. named after the inventor of the technique) method is a differeantial stain and one of the first steps in identifying procaryotic cells. This part of this laboratory work will help you Gram stain *known* gram-positive and gram-negative cultures.

Most bacteria has one of the three common shapes: coccus, rod, or spiral.

- **Coccus-shaped bacteria;** spherical or ovoid. Depending on the species bacterium may either appear as a single coccus or in one of following coccal arrangements: cocci in pairs (Diplococcus), cocci in chains, cocci in clusters.
- **Rod-shaped (bacillus) bacteria;** cylindrical and may appear single or in chains, or in pairs.
- **Spiral shaped bacteria;** may be found in one of the three following forms: ***Vibrio:*** *comma shaped; an incomplete spiral.* ***Spirillum:*** *a thick, rigid spiral.* ***Spirochete:*** *a thin, flexible spiral.*
- **Coccobasillus-shaped bacteria;** short bacilli with rounded ends resembling a coccus.

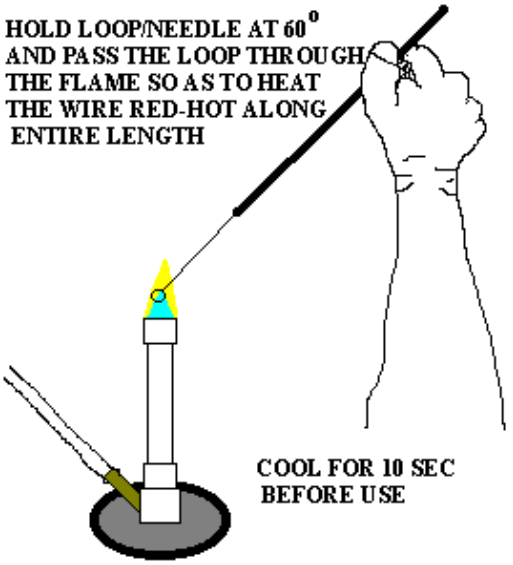
What should you do?

Materials and Methods:

Smear Preparation and Fixation

To prepare bacteria for staining (see the figures below), a portion of the bacterial isolate (i.e. picked up from a pure colony on solid medium) is spread as a thin layer or **SMEAR** on a clean glass slide. The thin bacterial film is allowed to **AIR DRY** and then it is **FIXED** to the glass to prevent it from washing-off during the subsequent staining procedure, by passing the slide briefly through a flame; this treatment makes the bacteria stick to the glass and also kills them.

STERILIZATION OF A LOOP BY FLAMING



ALWAYS FLAME THE LOOP IMMEDIATELY PRIOR TO USING IT FOR ANY PURPOSE.

Figure 1. Flaming of loop.
Place the loop in flame starting at the loop and move it through the flame so that the wire becomes red-hot along 4 to 6 cm of its length. Allow the wire to cool for about 10 seconds. Pick up the sample with the cooled loop and distribute it. Repeat the flaming/cooling procedure before laying the loop down on the desk.

PREPARATION OF A BACTERIAL SMEAR

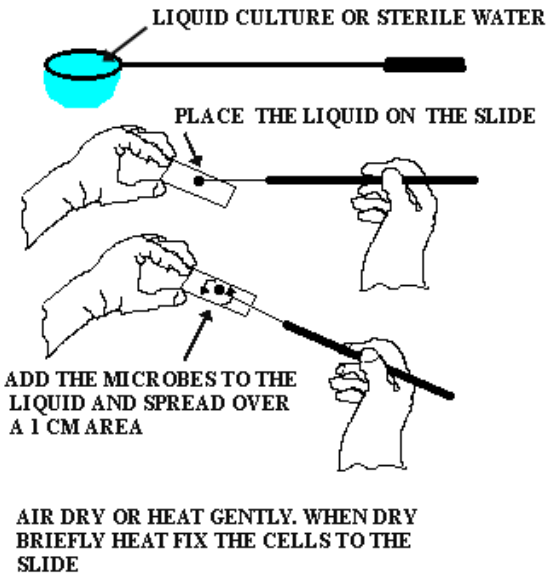


Figure.2. Preparation of the bacterial smear.

For a liquid sample, use a sterile/cooled loop to pick up some liquid sample in the loop. Place this liquid on the slide. ***For a dry sample*** (taking a colony from an agar plate), use your sterile/cool loop to first put a small drop of water on the slide. Then pick up a tiny bit of the microorganism sample from the source (colony, wound) with a sterile/cool loop and mix it into the liquid on the slide.

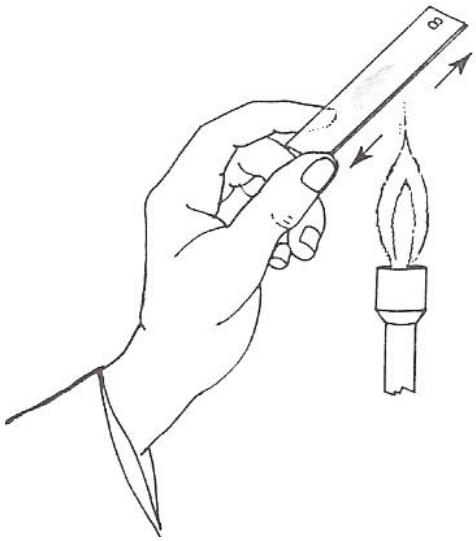


Figure.3. Fixation of the bacterial smear.

Air dry and fix it by heat.

Staining

Materials:

1. Overnight incubated agar plate cultures of *Escherichia coli, Staphylococcus aureus* and *Candida albicans.*
2. Microscope slides.
3. Inoculating loops and Bunsen burners.
4. Lens cleaning paper, immersion oil and microscope.
5. Gram staining set
6. Saline solution
7. Disinfectant solution in beakers
8. Lab. marker

Methods:

1. Prepare 2 sets of slides of each of the cultures.
2. Heat an inoculation loop to redness.
3. Cool it at the edge of the bacterial suspension tube
4. Put a drop of sterile saline solution on the glass slide.
5. Pick up a portion of bacterial isolate (i.e. from the pure colony on agar plate) on to the clean slide by using inoculation loop.
6. Reflame the inoculating loop to redness before putting it down for future use TO AVOID CONTAMINATION OF THE ENVIRONMENT.
7. Allow it to dry.
8. FIX your slide by heating: With the sample side up pass the dried slide through the flame of the Bunsen burner several times. Do not over heat. (This procedure will coagulate the protoplasm of the microorganism, causing them to stick -"be fixed"- to the slide).
9. Apply Gram staining technique to one both set of prepared slides.
10. Observe each slide with the low-power and oil immersion objectives.
11. Sketch a representative view of the samples using oil immersion objective **in your results** section.
12. Discard the slides in the disinfectant container as indicated and wipe the objectives.
13. **In your discussion section:**
    a. *Compare your known and unknown cultures. Did you identify them easily?*
    b. *What color is Gram negative?*
    c. *What color is Gram positive?*
    d. *Which staining technique is ideal for identifying mixed cultures?*
    e. *Compare your Gram staining technique with wet mount technique.*

Procedure of the Gram Staining Technique:

1st step: the fixed smear is stained with crystal violet for 30 seconds.
(At this step, Gram positive bacteria stains purple or dark blue)
Wash it with water
2nd step: iodine solution is applied for 30 seconds.
(This step increases the interaction of main dye -mordan- with the cell wall.)
Wash it with water
3rd step: decolorizing with ethanol for 30 seconds.
(At this step, Gram positive: retain the crystal violet, Gram negative lose it.)
Wash it with water
4th step: counterstaining with safranin for 30 seconds.
Wash it with water

As a result, at the last step, **Gram positive stays purple or dark blue** while **Gram negative stain pink to red.**

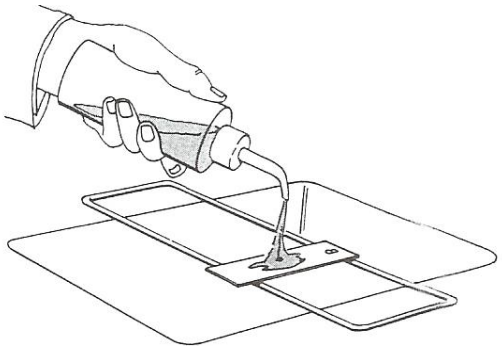


Figure 3. Adding stain to the fixed bacterial smear.
After the bacterial smear has been heat-fixed to the slide, lay it on the slide-support rack. Carefully drop the appropriate staining solution onto the smear so as to cover it entirely. Allow it to sit for 30 to 60 seconds. Then tip the slide so the excess stain drops into the reservoir. Gently run a spray of deionized water over the smear, washing off any remaining stain. Finally, dry the smear in the air. Examine under the microscope, first using the 10X lens to locate areas of stained material, and then the 40X to focus, and finally place a drop of oil on the sample and rotate the oil-immersion lens to the slide.

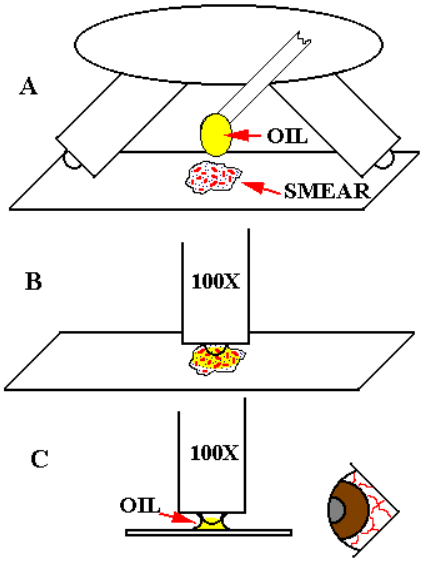
The stain is then washed off with a gentle stream of water and the slide is **BLOTTED DRY**. The dried, stained smear is then viewed with the microscope. Your instructor will demonstrate these techniques.

Microscopic Examination

Observing stained bacteria using the 100X objective

View the bacteria first through the lower power fields (10x10, 10x40) and then through the oil immersion objective (10x100) (see the figure below).

1. start on low power field (10X10 mag. power)
2. focus
3. move to high power field (10x40 mag. power)
4. focus
5. move to oil immersion field (10x100 mag. power)
6. focus
7. note cellular morphology;
    a. Draw a circle in your lab notebook for each sample. Label each circle with the **SAMPLE**, **STAIN**, and **MAGNIFICATION**, and draw the sample as you see it under the **100X OBJECTIVE.**
    b. Describe the cells in the pure cultures as to their **FORM** (morphology), **SIZE,** and **ARRANGEMENT** of cells in a group.
    c. **In your discussion section:**
        - How can you differentiate *E.coli, Staphylococcus aureus, Streptococci species and Candida albicans* from each other by this staining technique?



LABORATORY REPORT

DATE:

TITLE:

INTRODUCTION (Purpose of the study):

MATERIALS AND METHODS:

RESULTS:

DISCUSSION/CONCLUSION:

4.1. Culture Methods in Diagnostic Microbiology

Inoculation Techniques and Incubation Conditions

What should you know?

Inoculation techniques (see the figures below):

1. Spread plate technique (mainly used for antimicrobial susceptibility testing)
2. Streak plate technique (mainly used to obtain pure colony for further diagnostic examinations)
3. Pour plate technique (mainly for counting the number of colonies in the specimen)

However, the aim or main principle of the diagnostic culture methods is to demonstrate the presence of infectious agent (i.e. bacteria) in the clinical specimen by isolating the agent as a pure colony on agar plate.

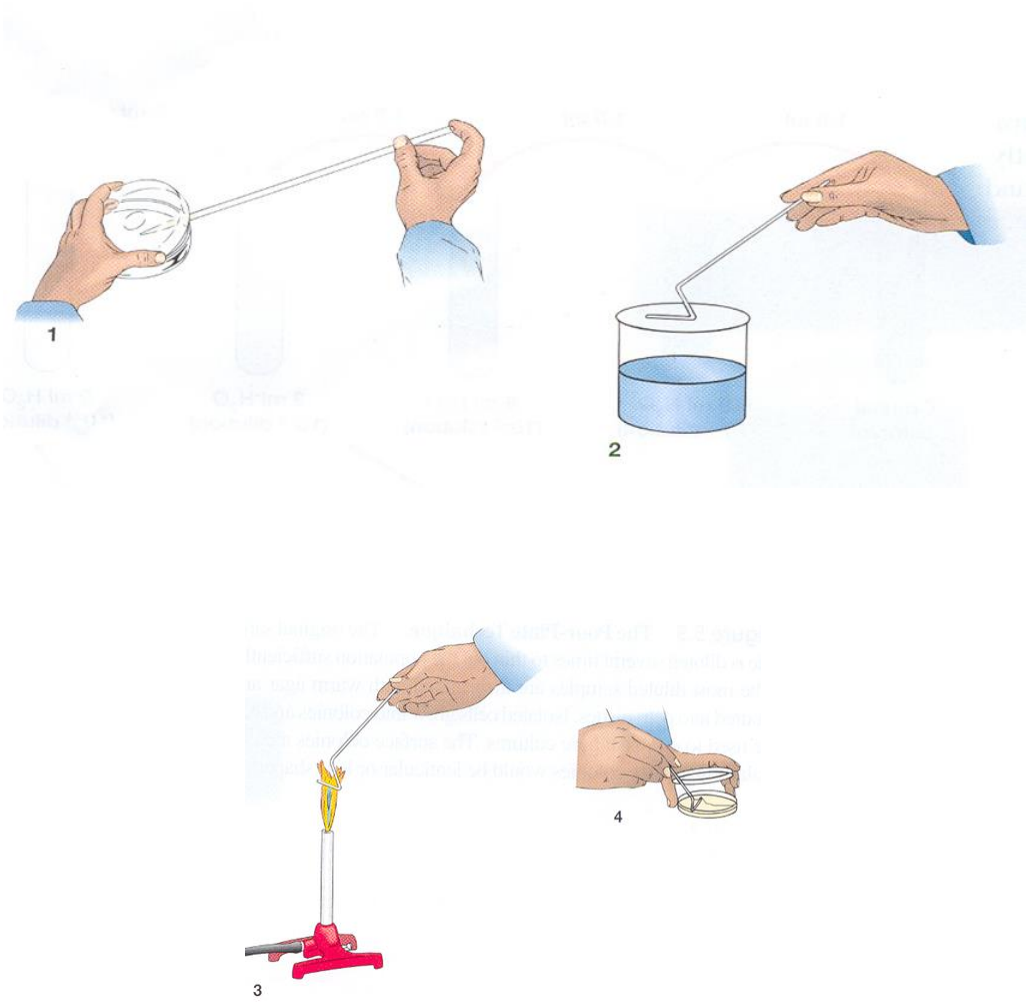


Figure.1. Spread plate technique.

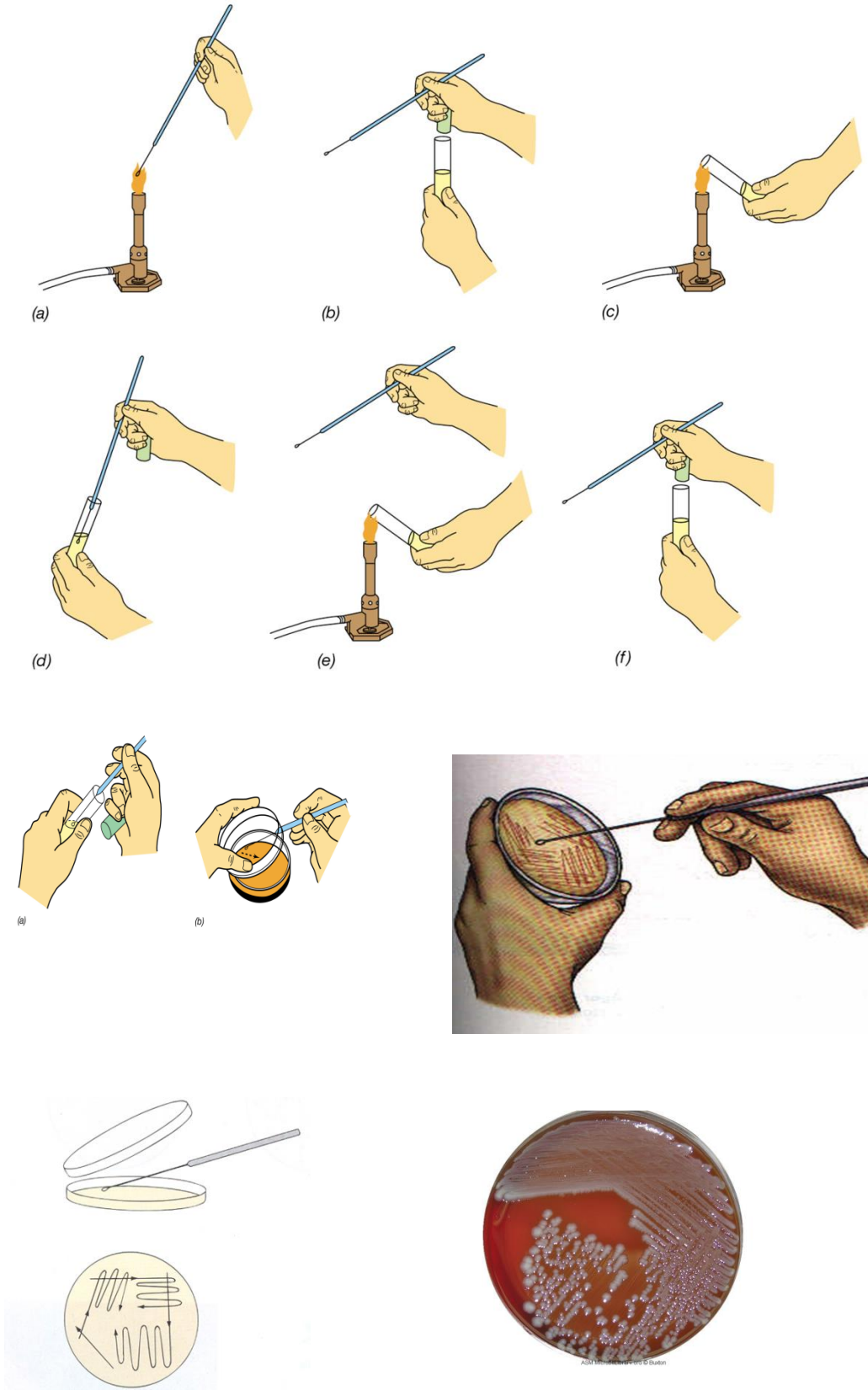


Figure.2. Streak plate technique.

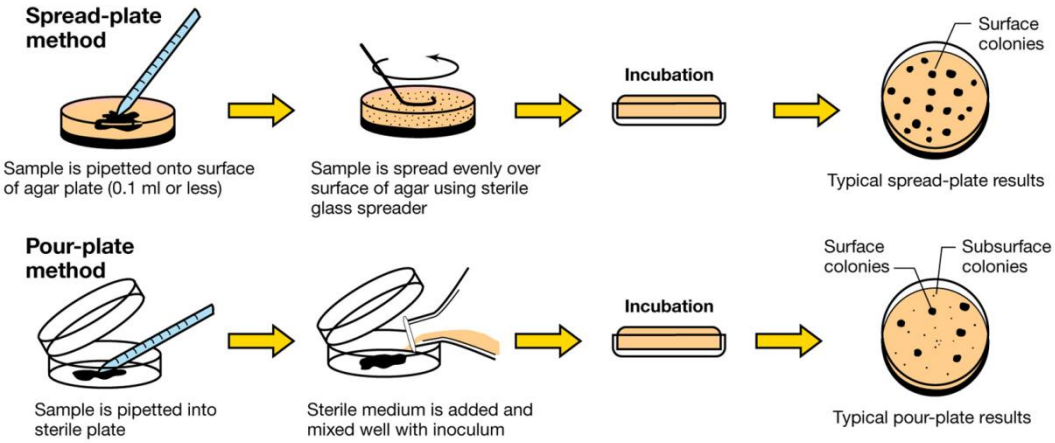


Figure.3. Spread-plate *versus* pour plate technique.

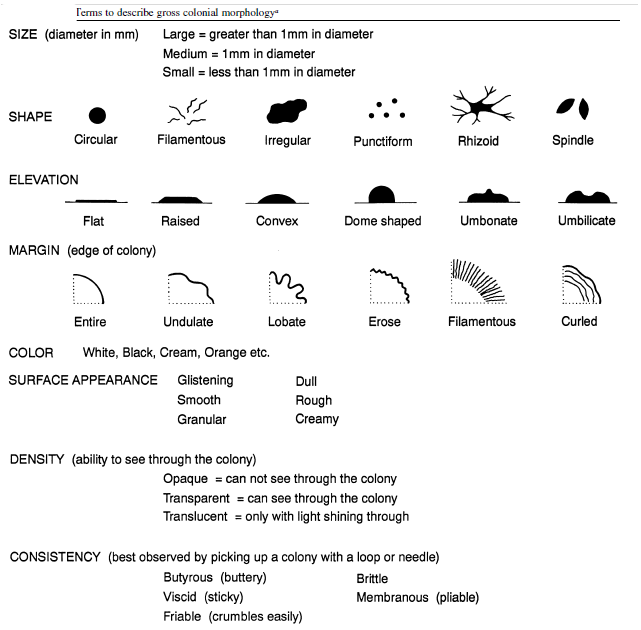


Figure.4. Typical colony morphologies of various bacteria on agar plate (**Reference:** *Garcia L.S., Isenberg H.D. Clinical Microbiology Procedures Handbook, ASM Press, Washington, 2010.*).

Incubation Conditions

- **Incubation time for bacterial cultures;**
  - Normal bacterial culture reports are given after 24-48 hours.
  - Anaerobic cultures are routinely held for 5-7 days.
  - For *Actinomyces*; 3 weeks.
  - For *Mycobacteria*; 2-4 weeks.
- **Incubation conditions according to heat requirements;**
  - Cardinal temperatures;
    - **minimum temperature**
    - **optimum temperature**
    - **maximum temperature**
  - **Psychrophiles**
  - ***Mesophiles***
  - **Thermophiles**
  - **Hyperthermophiles**
  - Most bacteria that have adapted to humans are mesophiles that grow best near human temperature (37 °C)
  - Diagnostic laboratories incubate cultures at 35 °C
  - Fungal cultures are incubated at 30 °C
  - The ability to grow at room temperature (25 °C) or at an elevated temperature (42 °C) is used as a differential, selective or enrichment characteristic in diagnostic laboratories for some bacteria.
- **According to oxygen (O_2) requirements:**
  - **Obligate anaerobes**; cannot grow in the presence of oxygen (e.g. *Clostridium perfringens*)
  - **Aerotolerant anaerobes, facultative aerobes**; can survive in the presence of oxygen but do not use oxygen in their metabolism (e.g. **certain *Clostridium* spp**.)
  - **Obligate aerobes**; require oxygen for growth
  - **Facultative anaerobes;** grow either with or without oxygen (e.g. ***Escherichia coli***)
  - **Microaerophilic;** require reduced level of oxygen (5-6%) (e.g**. *Helicobacter* spp.*, Campylobacter* spp.**)
  - **Capnophilic;** grow best when enriched with extra carbon dioxide(5-10%) (e.g. ***Haemophilus influenzae***)
  - **Therefore, athmospheric incubation conditions for growth can be;**
    - **Aerobic**
    - **Anaerobic** (in special jars)
    - **Capneic** (%5-10 Carbon dioxide in special jars or incubators)
    - **Microaerophilic** (in special jars or commercially available pouches)
  - **Air**
    - 21% oxygen
    - 1% carbon dioxide
  - In a condition with 10% carbon dioxide, oxygen content decreases to 18% which is enough for obligate aerobes.
- **According to pH requirements;**
  - **Acidophiles**: below pH 5 (many fungi)
  - **Alkaliphiles**: above pH 9 (*Vibrio*)
  - **Neutral pH**: most pathogens

Types of Culture Media

- **A basic culture medium includes;**
  - Water
  - Carbon
  - Nitrogen, sulphur, phosphorus, oxygen, hydrogen
  - Metal ions, vitamins
- **Major categories of culture media:**
  - According to the composition of the ingredients;
    - Defined media (ingredients in known quality and quantity)
    - Complex (undefined) media (ingredients in unknown quality and quantity)
  - According to physicochemical state of the medium;
    - Solid (phase) media (agar plate); includes agar, solidify at 45 ^{o}C, 1.5-2% agar (w/v),
    - Semi-solid (phase) media ; includes agar as 0.5% (w/v)
    - Liquid (phase) media (broth)
  - According to the utilization functions;
    - Basal media
    - Supportive media
    - Enrichment media (for fastidious bacteria)
    - Differential media
    - Selective media
    - Transport media
  - According to the utilization functions;
    - **Basal media**
      - **Nutrient broth**
        - » beef extract
        - » peptone
        - » NaCl
        - » water
    - **Supportive media**
      - contains nutrients that support growth of most non-fastidious bacteria without giving any particular organism a growth advantage
      - %5 SBA ; **nutrient agar.**
    - **Enrichment media** (for fastidious bacteria)
      - contain specific nutrients required for the growth of particular bacterial pathogens that may be present alone or with other bacterial species in a patient specimen; B**lood Agar, Chocolate Agar.**
    - **Differential media**
      - employ some factor/s that allow/s colonies of one bacterial species or type to exhibit certain metabolic or cultural characteristics that can be used to distinguish them from others; **Bile-Esculin Agar, Hectoen Enteric Agar, MacConcey Agar**
    - **Selective media**
      - contain one or more agents that are inhibitory to all organisms except those being sought; select for the growth certain bacteria; **Campy-Blood Agar**
    - **Transport media**
      - When a delay between collection of the specimen and culturing the specimen is necessary a transport medium is used:
        - » Stuart broth (urogenital specimen, wound culture)
        - » Cary-Blair transport (feces) media

Enumeration of colonies on/in culture media

Enumeration guidelines

Report	If the observation is growth
Isolated from broth only	In broth only
Scant growth	One to five colonies. For one colony of skin microbiota, may wish to add note regarding possible contamination.
1+ or few	In first quadrant only, ignoring a few colonies in the second quadrant
2+ or moderate	Up to second quadrant, ignoring a few colonies in the next quadrants
3+ or numerous	Up to third quadrant, ignoring a few colonies in the fourth quadrant
4+ or numerous	Up to fourth quadrant
Number + CFU/ml	For urine and quantitative cultures
Number + CFU	For intravascular catheter tips

Reference: *Garcia L.S., Isenberg H.D. Clinical Microbiology Procedures Handbook, ASM Press, Washington, 2010.*

Evaluation of colony morphology on culture media

Colonial morphology on primary media[a]

Organism(s)	Morphology on:		
	BAP or CNA[b]	CHOC	MAC/EMB[c]
Escherichia coli	Gray, mucoid, flat or convex, not swarming, may be beta-hemolytic[d]	Same as BAP	Pink/dark center and may have green sheen
Proteus spp.	Flat, gray, spreading[d]	Same as BAP	Colorless
Pseudomonas aeruginosa	Flat, gray-green, rough, may have spreading margins, metallic sheen, may be extremely mucoid[d]	Same as BAP	Colorless
Neisseria gonorrhoeae	Inhibited	Small, gray, entire, sticky	Inhibited
Neisseria meningitidis	Medium to large, creamy and gray, alpha-hemolytic[d]	Same as BAP, no hemolysis	Inhibited
Haemophilus spp.	Inhibited	Gray, raised, smooth, may be mucoid	Inhibited
Moraxella catarrhalis	Whitish, medium to large, raised or dome shaped[d]	Same as BAP	Inhibited
Staphylococcus aureus	Large, convex, white-yellow, creamy, opaque, may be beta-hemolytic	Same as BAP, no hemolysis	Inhibited/may be pinpoint
Coagulase-negative staphylococci	White-gray, raised, creamy	Same as BAP	Inhibited
Beta-hemolytic streptococci	Pinpoint to medium, zone of beta-hemolysis (clear zone) translucent, dull, gray	Same as BAP, no hemolysis	Inhibited
Viridans group streptococci	Pinpoint to medium, white-gray, caramel odor, alpha-hemolysis	Same as BAP	Inhibited
Enterococcus spp.	Gray, medium, usually no hemolysis	Same as BAP	Inhibited/may be pinpoint
Streptococcus pneumoniae	Umbilicate, alpha-hemolysis, transparent, may be mucoid, flattened, or teardrop shaped	Same as BAP	Inhibited
Listeria monocytogenes	Whitish gray similar to group B streptococcus, flat, narrow zones of beta-hemolysis	Same as BAP, no hemolysis	Inhibited
Corynebacterium spp.	White, dry, may be sticky	Same as BAP	Inhibited
Yeast cells	White, creamy, bread odor, "feet" extending from colony	Same as BAP	Inhibited/pinpoint

Reference: *Garcia L.S., Isenberg H.D. Clinical Microbiology Procedures Handbook, ASM Press, Washington, 2010.*

What should you do?

Materials and methods:

Materials:

1. Broth culture of *E. coli*.
2. A loop.
3. A solid agar media (i.e. % 5 SBA, EMB Agar, MH Agar, SS Agar).
4. Micro-incinerator.

Method:

1. By applying sterile technique, pick up a loopful of broth culture.
2. Streak the %5 SBA medium as shown in the figure.
3. Repeat step 1 and 2 for the other agar media (i.e. EMB Agar, MH Agar, SS Agar)
4. Inoculate the plate at 35 ^{o}C for 16-18 hours.
5. Evaluate the colony morphology on the next day.
6. Write your assessments in the "**discussion section"** of the laboratory report:
    a. *on the success of obtaining pure colonies at the fourth region of the plate*
    b. *the colony morphology of E. coli on different media and compare the results*.

LABORATORY REPORT

DATE:

TITLE:

INTRODUCTION (Purpose of the study):

MATERIALS AND METHODS:

RESULTS:

DISCUSSION/CONCLUSION:

5.1. Identification Methods in Diagnostic Microbiology

Identification of *Enterobacteriaceae*

What should you know?

- *Enterobacteriaceae* is a large family of bacteria that includes most heterogeneous collection of medically important gram-negative rods with more than 40 genera and 150 species.
- The genera have been classified based on *biochemical properties,* antigenic structure, nucleic acid hybridization and sequencing.
- Fewer than 20 species are responsible for more than 95% of the infections.
- These bacteria can be found in environment, and also consists part of normal flora of humans and most animals.
- They cause 30 to 35% of septicemias, more than 70% of urinary tract infections and many intestinal infections.
- The steps in the identification of an unknown bacteria are;
    1. **to isolate the microorganism in pure culture** from the clinical or environmental specimen (i.e. *using selective and differential media such as MacConkey Agar, Endo Agar, EMB Agar or SS Agar medium*) (Practice.4.1.),
    2. **to determine the colony morphology** of the isolate (Practice.4.1.),
    3. **to determine Gram-staining characteristics** of the isolate (Practice.3.1.),
    4. **to determine oxidase status** of the isolate (*i.e. especially useful in distinguishing the oxidase negative Enterobacteriaceae from Pseudomonadaceae that are oxidase positive*) **(Practice.5.1**.)
    5. **to do additional biochemical tests** necessary for definite identification. (**Practice.5.1.**)
- In a microbiology laboratory, certain types of tests are routinely used for the identification of an unknown bacterium.
- In this laboratory work, selected tests and procedures will be performed to identify bacterial isolates belonging to *Enterobacteriaceae.*

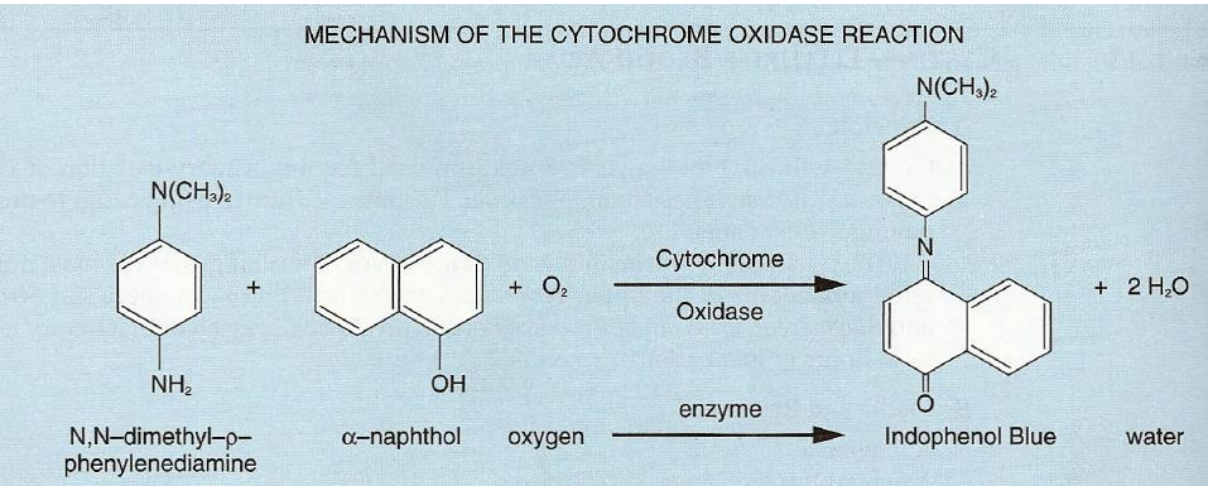
Phenotype (Biochemical) tests
(Oxidase, TSI, Urease, Motility, IMViC tests)

Cytochrome oxidase test

Principle

The cytochromes are iron-containing hemoproteins that form the last phase in the aerobic respiration by transferring electrons (hydrogen) to oxygen, with the formation of water. The cytochrome system can be shown in aerobic, or microaerophilic, and facultatively anaerobic organisms, so the **oxidase test** is important in identifying organisms that either lack the enzyme or are obligate anaerobes. The test is especially used in screening colonies suspected of being one of the ***Enterobacteriaceae*** **(all negative)** and in identifying colonies that belong to other genera such as *Aeromonas, Pseudomonas, Neisseria, Campylobacter,* and *Pasteurella* (**positive**).

The cytochrome oxidase test contains certain reagent dyes, such as p-phenylenediamine dihydrochloride, that substitute for oxygen as artificial electron acceptors. In the reduced state, the dye is without colour; however, in the presence of cytochrome oxidase and atmospheric oxygen, p-phenylenediamine is oxidized, and produces indophenol blue.



Media and Reagents

Tetramethyl-p-phenylenediamine dihydrochloride, 1% (Kovac's reagent)

Quality Control

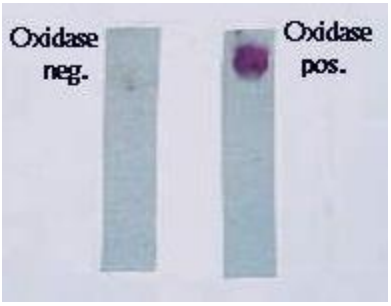
A. **Positive control-*Pseudomonas aeruginosa***
B. **Negative control-*Escherichia coli***

Procedure

The test is frequently performed by the indirect paper strip procedure, in which either a few drops of the reagent are added to a filter paper strip or commercial disks or strips impregnated with dried reagent are used. A suspected colony is picked up with a loop and smeared into the reagent zone of the filter paper.

Results-Interpretation

Bacterial colonies having cytochrome oxidase activity develop a deep blue colour at the inoculation site within 10 seconds. **Any organism producing a blue colour in the 10- to 60-second period must be further tested because it probably does not belong to the *Enterobacteriaceae*.**



Attention: Stainless steel or Nichrome inoculating loops or wires should not be used for this test because surface oxidation products formed when flame sterilizing may result in false-positive reactions.

Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Biochemical tests in Triple Sugar Iron (TSI) Agar Medium

Principle

Triple Sugar Iron (TSI) agar slants differentiate bacteria on their ability to **ferment glucose, lactose, and/or sucrose** and on their ability to **reduce sulfur to hydrogen sulfide**. TSI agar slant in a test tube contains agar, a pH-sensitive dye (phenol red), glucose (0.1%), lactose (1.0%) and sucrose (1.0%), as well as sodium thiosulfate, ferrous (iron) sulfate and peptone (this is an alternative energy source that is not fermentable). All of these ingredients are mixed together and allowed to solidify in the test tube **at a slanted angle**. The slanted shape of this medium provides **an array of surfaces** that are either exposed to oxygen-containing air in varying degrees (**an aerobic environment**) or not exposed to air (**an anaerobic environment**).

Many of the enteric organisms will ferment glucose with the production of acids which will change the color of the medium in the *butt and along the slant* from *red to yellow because of a reduction in the pH* (**within the first few hours**). However**, since the glucose is present in small amounts (0.1%),** the supply is soon exhausted and the organisms growing on the surface of the *slant in the presence of oxygen* are **forced to catabolize peptones and amino acids** for their energy supply. Alkaline end-products are produced from these substances which revert the pH of the slant to *an alkaline pH* and thus change the color of the *agar slant back to red* (**after 18-24 hours**). Since metabolism is progressing at a slower rate in the butt, this reversion does not usually take place in the butt until 48 hours or longer.

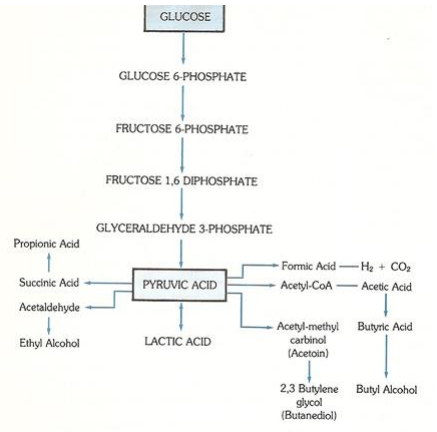
If the glucose is **metabolized under anaerobic conditions,** the **gas** produced will be seen as *bubbles or cracks in the agar butt*. Under anaerobic conditions (as occur toward the bottom of the tube) some bacteria use H^+ as an electron acceptor and reduce it to **hydrogen gas.** This is not very soluble and may accumulate as bubbles along the inoculation track, between the agar and the glass, or in the fluid which accumulates at the bottom of the slant.

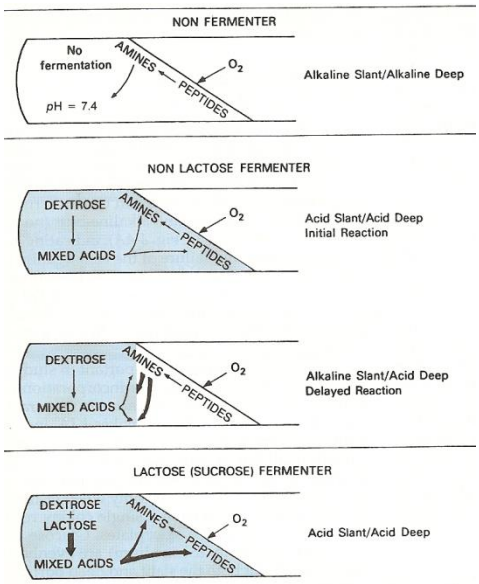
Hydrogen production may lift the agar from the butt of the tube or fracture the agar. Carbon dioxide, if produced, may not show as bubbles because it is far more soluble in the medium.

Some bacteria utilize thiosulfate anion as a terminal electron acceptor, reducing it to sulfide. If this occurs, the **newly-formed hydrogen sulfide** (H_2S) reacts with ferrous sulfate in the medium to form **ferrous sulfide**, which is visible as a **black precipitate.** The blackening of the medium is almost always observed in the butt (bottom) of the medium.

If **hydrogen sulfide is formed** during growth, a *gray or black streak* of **iron sulfide** is seen originating where the *inoculating needle entered and throughout the agar butt*. Blackening of the butt due to H_2S production **may mask the acid reaction (yellow) in the butt**. *Salmonella enterica* serovar Typhi may result in blackening of the medium **at the interface** of butt and slant.

If there is **no fermentation**, then there should be **no change** (except for growth on the slanted surface).





Media and Reagents

Triple Sugar Iron (TSI) agar -slant- medium.

Quality Control

A. **Positive control:**

Glucose: Salmonella

Lactose/Sucrose: Escherichia coli

Hydrogen sulfide (H_2S): Citrobacter freundii

B. **Negative control *(non-fermentative)*:** *Pseudomonas aeruginosa*

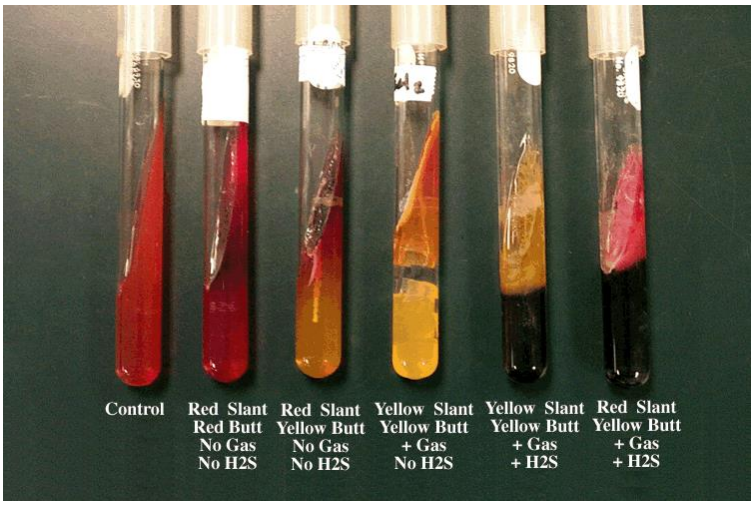
Procedure

Use an inoculating needle to make a stab inoculation into one of the TSI slants. Use an inoculating loop to inoculate the slanted portion of the same TSI slant. Incubate at 35 ^{o}C for 24 hours. After overnight incubation, obtain your slants from the incubator and observe the color and gas production.

Results-Interpretation

Possible TSI Reactions

Slant/Butt/Gas	Glucose	Lactose	Sucrose	H_2S
Red/Red/None	-	-	-	-
Red/Yellow/None	A	-	-	-
Red/Yellow/Gas	AG	-	-	-
Yellow/Yellow/Gas	AG	AG	?	-
Yellow/Black/Gas	AG	-	AG	+
Red/Black/Gas	AG	-	-	+

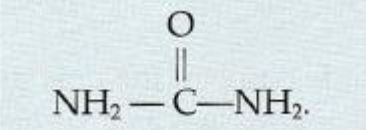


Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

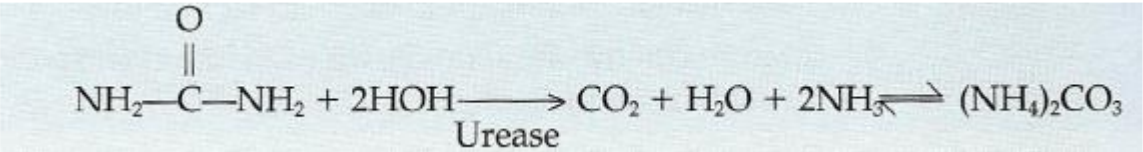
Urease test

Principle

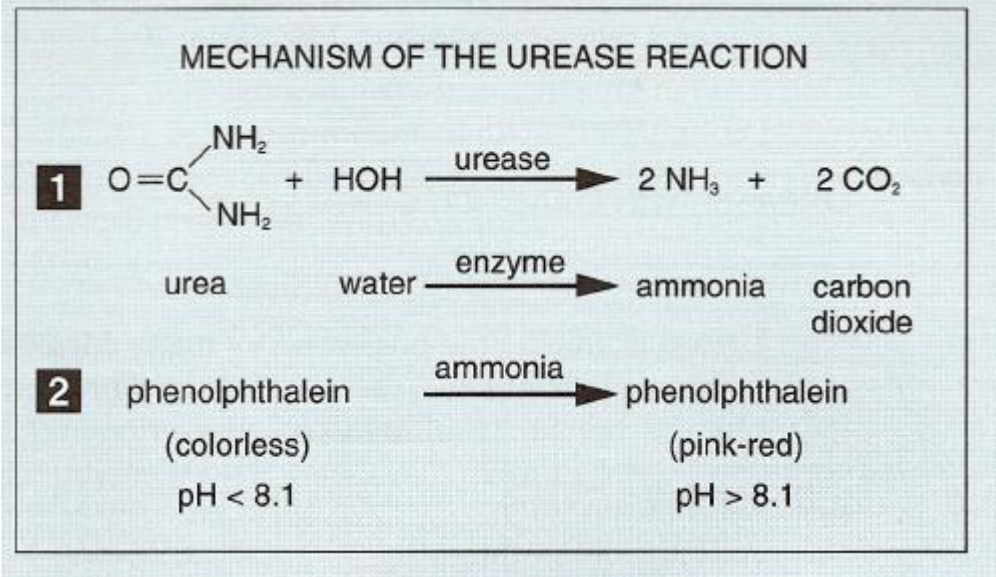
Urea is a diamide of carbonic acid as shown in the formula:



All amides are easily hydrolyzed with the release of ammonia and carbon dioxide. Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea by the following chemical reaction:



The ammonia reacts in solution to form ammonium carbonate, resulting in alkalinization and an increase in the pH of the medium.



Media and Reagents

In the detection of urease activity, Stuart's urea broth and Christensen's urea agar are the most frequently used media in clinical laboratories.

STUART'S UREA BROTH	
Yeast extract	0.1 g
Monopotassium phosphate	9.1 g
Disodium phosphate	9.5 g
Urea	20 g
Phenol red	0.01 g
Distilled water to	1 L

Final pH = 6.8

CHRISTENSEN'S UREA AGAR	
Peptone	19 g
Glucose	19 g
Sodium chloride	5 g
Monopotassium phosphate	2 g
Phenol red	0.012 g
Urea	20 g
Agar	15 g
Distilled water to	1 L

Final pH = 6.8

Quality Control

As a positive and negative control, organisms that react accordingly should be tested for each new batch of medium:

A. **Positive control**: *Proteus* species
B. **Positive control** (**weak**): *Klebsiella* species
C. **Negative control**: *Escherichia coli*

Procedure

A loopful of pure culture of the test organism should be inoculated to the broth or the surface of the agar slant by streaking. Incubation should be done at 35 °C for 18 to 24 hours.

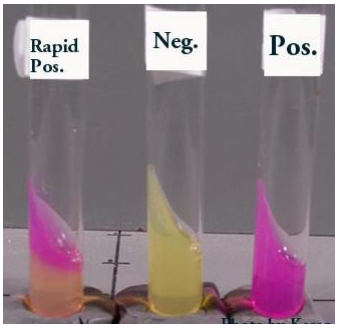
Results

Interpretation:

Rapid splitter organisms hydrolyze urea within 1 or 2 hours whereas slow splitters require 3 or more days.

The reactions are as follows:

1. *Stuart's broth*:
   Color of the whole medium turns into red which indicates alkalinization and urea hydrolysis.
2. *Christensen's agar*:
   Rapid urea splitters (*Proteus* species): color of the whole medium turns into red
   Slow urea splitters (*Klebsiella* species): red color initially in slant only, gradually color of the whole medium turns into red
3. *No urea hydrolysis*: original medium color (yellow) does not change.



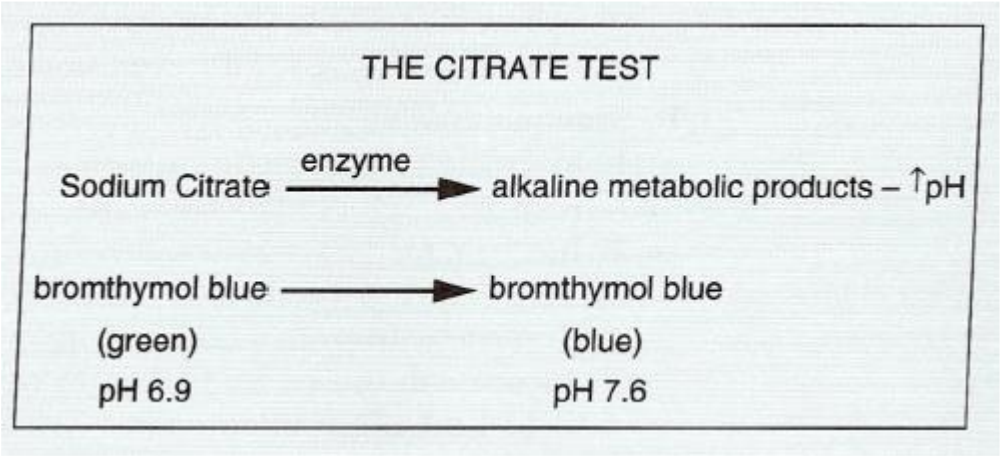
Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Citrate utilization test

Principle

Sodium citrate is a citric acid salt, a simple organic compound that is among metabolites in the tricarboxylic acid cycle (Krebs cycle). Some bacteria can use citrate as the sole source of carbon and obtain energy in this way which is different than fermentation of carbohydrates. Presence or absence of citrate utilization is a criterion in the identification of many members of the *Enterobacteriaceae*. Citrate utilization test is done in a medium that does not include protein and carbohydrates as sources of carbon.

Citrate utilization of a test bacterium is detected in test medium by the production of alkaline by-products. In the test medium, sodium citrate, an anion, is included as the sole source of carbon, and ammonium phosphate as the sole source of nitrogen. Bacteria that can use citrate can also extract nitrogen from the ammonium salt, with the production of ammonia (NH^+), leading to alkalinization of the medium from conversion of the NH^{2+}_3 to ammonium hydroxide (NH_40H). Bromthymol blue -*yellow below pH 6.0 and blue above pH 7.6*- is the indicator.



Media and Reagents

Most commonly used citrate medium is based on the formula of Simmons. The medium is poured into a tube on a slant. The formula of Simmons citrate medium is shown below:

SIMMONS CITRATE MEDIUM	
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2 g
Magnesium sulfate	0.20 g
Agar	15 g
Bromthymol blue	0.08 g
Distilled water to	1 L

Quality Control

Each new batch of medium should be tested with a positive- and a negative-reacting organism. The following species are suggested controls:

A. **Positive control**: *Enterobacter aerogenes*

B. **Negative control**: *Escherichia coli*

Procedure

A pure colony is picked from the surface of an isolation medium and inoculated as a single streak on the slant surface of the citrate agar tube. The tube is incubated at 35°C for 24 to 48 hours.

Results

Interpretation:

Either the presence of deep blue color or visible colonial growth along the inoculation streak line within 24 to 48 hours indicate a positive test result. In the latter case, if incubation is continued for an additional 24 hours, a blue color usually develops.

Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Motility test

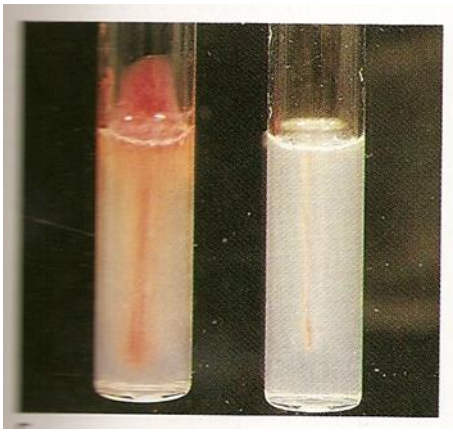
Principle

Bacteria move via flagella. Bacterial motility can be observed directly by plating a drop of culture broth medium on a microscope slide and examining it under a microscope. Hanging-drop slides are available for observation under higher magnification without the risk of lowering the objectives onto the contaminated drop. This technique is appropriate for motility detection in bacteria that do not grow well in semisolid agar media. However, *Enterobacteriaceae* grow well in semisolid agar media.

Media and Reagents

Motility media have 0.4% or lesser agar concentrations. At higher concentrations, the gel is too stable and does not allow the organism to spread freely. Motility test medium supports the growth of most fastidious bacteria and has a crystal clear appearance.

MOTILITY TEST MEDIUM (EDWARDS and EWING)	
Beef extract,	3 g
Peptone,	10 g
Sodium chloride,	5 g
Agar,	4 g
Bromthymol blue	0.08 g
Distilled water to	1 L
Final pH = 7.3	



Quality Control

Each new batch of medium should be tested with a motile and a non-motile organism. The following species are suggested controls:

A. **Positive control**: *Escherichia coli*

B. **Negative control**: *Klebsiella pneumonia*

Procedure

A pure colony is inoculated as a vertical line dipped from the surface towards the bottom of the test medium tube. The tube is incubated at 35°C for 24 hours.

Results

Interpretation:

If a diffuse zone of growth spreading horizontally from the line of inoculation is observed by making a macroscopic examination of the medium, the test bacterium is positive for motility.

Reference: *Modified from Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Indole test

Principle

Indole is a metabolic degradation product of tryptophan (amino acid). A bacterium that possess tryptophanase is capable of degrading (i.e. hydrolyzing and deaminating) tryptophan. This process ends up in the production of indole, pyruvic acid and ammonia.

When indole reacts with *p*-dimethylaminobenzaldehyde (i.e. active chemical in Kovac and Ehrlich reagents), a red complex is formed. Test medium should be rich in tryptophan.

Media and Reagents

TRYPTOPHAN BROTH (%1)	
Peptone or pancreatic digest of casein (trypticase)	2 g
Sodium chloride,	0.5 g
Distilled water to	100 mL
Final pH = 7.3	

KOVAC REAGENT	
Pure amyl or isoamyl alcohol	150 mL
p-dimethylaminobenzaldehyde	10 g
Concentrated HCl	50 mL

Quality Control

Each new batch of medium should be tested with a positive- and a negative-reacting organism. The following species are suggested controls:

A. **Positive control**: *Escherichia coli*

B. **Negative control**: *Klebsiella pneumonia*

Procedure

Test medium is inoculated with the test bacterium and incubated at 35°C for 18 to 24 hours.

Results

Interpretation:

If a red color develops within seconds at the interface of reagent and medium, it indicates the presence of indole; therefore test result is positive.

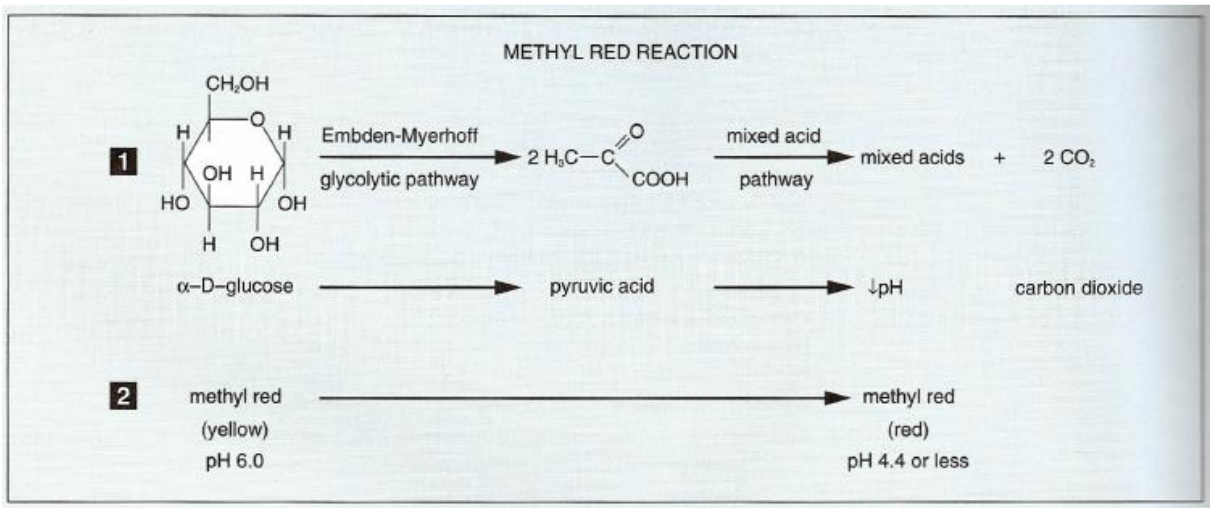
Reference: *Modified from Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Methyl-red (MR) test

Principle

Methyl red acts as a comparatively insensitive (i.e. detects acid is considerably lower than the pH for other indicators used in bacteriologic culture media) colorimetric indicator between pH 6.0 (yellow) and 4.4 (red). Therefore, to produce a color change, the test organism must produce large quantities of acid from the carbohydrate substrate.

The methyl red test acts as a quantitative test for acid production, because only the organisms that produce strong acids (lactic, acetic, formic) from glucose through the mixed acid fermentation pathway may give a positive result. Many species of the *Enterobacteriaceae* may produce sufficient quantities of strong acids that can be detected by methyl red indicator during the initial phases of incubation. However, only the organisms that can maintain this low pH after prolonged incubation (48 to 72 hours), overcoming the pH-buffering system of the medium, is called methyl red positive.



Media and Reagents

The medium most frequently used is methyl-red/Voges-Proskauer (MR/VP) broth, as formulated by Clark and Lubs. The same medium also used in the performance of the Voges-Proskauer test.

MR/VP Broth	
Polypeptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g
Distilled water to	1 L
Final pH = 6.9	

MR pH Indicator	
Methyl red, 0.1 g in 300 mL of 95% ethyl alcohol	
Distilled water	200 mL

Quality Control

Positive and negative controls should be run after preparation of each new lot/batch of medium and reagent. Suggested controls are:

A. Positive control: *Escherichia coli*
B. Negative control: *Enterobacter aerogenes*

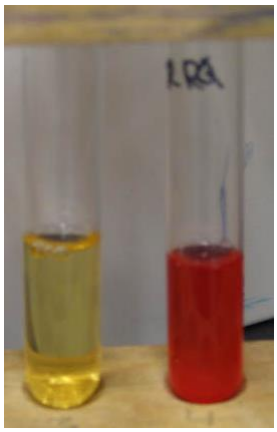
Procedure

A pure culture of the test organism should be inoculated to MR/VP broth. Incubation should be at 35°C for 48 to 72 hours (no fewer than 48 hours). After incubation, add 5 drops of the methyl red reagent to the broth.

Results

Interpretation

Development of a red color in the surface of the medium indicates acid production at pH=4.4 that corresponds to a positive test result. However, some organisms may produce smaller quantities of acid from the test substrate, an intermediate orange color between yellow and red may develop. Such a result is negative.



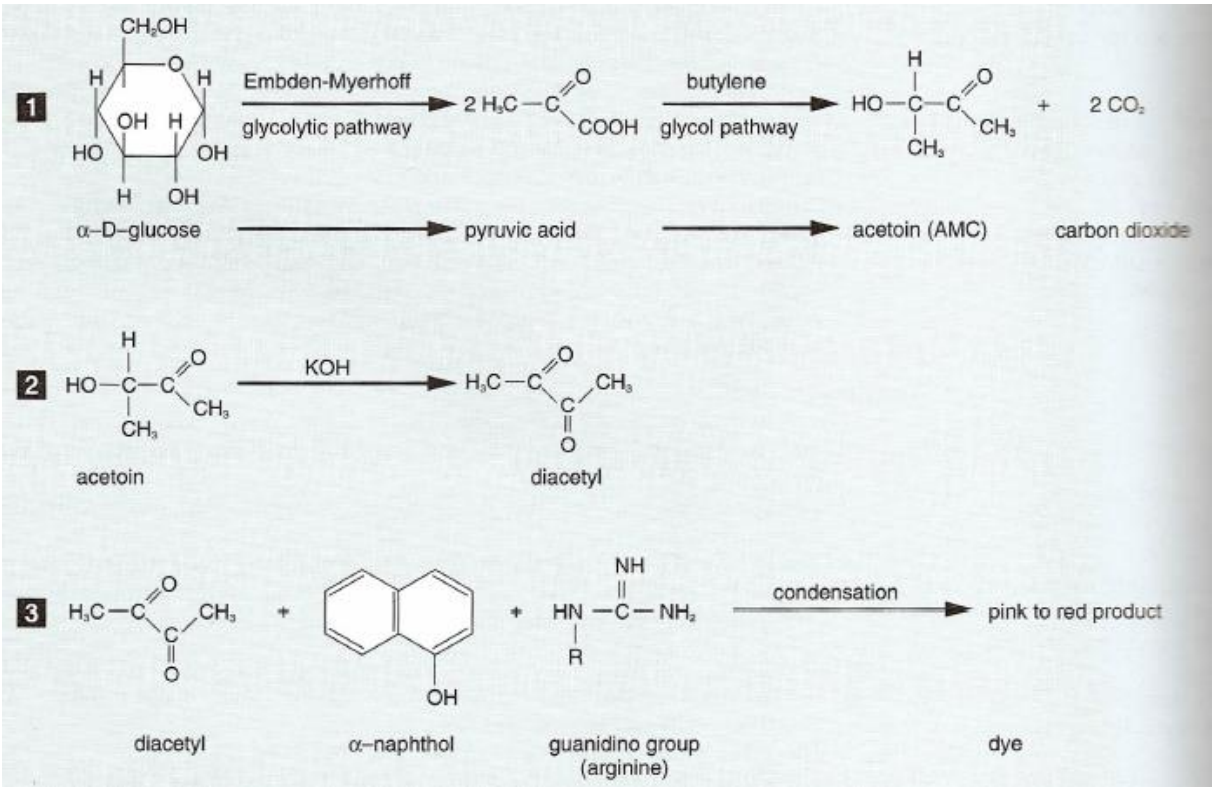
Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Voges-Proskauer (VP) test

Principle

Voges-Proskauer is a double eponym, named after two rnicrobiologists who described the red color reaction produced by appropriate culture media after treatment with potassium hydroxide. It was later understood that the active product in the medium formed by bacterial metabolism is acetylmethyl carbinol, a product of the butylene glycol pathway.

Pyruvic acid, the pivotal compound formed in the fermentative degradation of glucose, is further metabolized through various metabolic pathways, depending on the differences in bacterial enzyme systems. One of the pathways results in the production of acetoin (acetyl methyl carbinol), a neutral-reacting end product. *Klebsiella-Enterobacter-Hafnia-Serratia* group of organisms produce acetoin as the chief end product of glucose metabolism and form smaller quantities of mixed acids. In the presence of atmospheric oxygen and 40% potassium hydroxide, acetoin is converted to diacetyl, and α-naphthol acts as a catalyst to form a red complex.



Media and Reagents

The medium most frequently used is methyl-red/Voges-Proskauer (MR/VP) broth, as formulated by Clark and Lubs. The same medium also used in the performance of the metyhl red test.

MR/VP Broth	
Polypeptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g
Distilled water to	1 L
Final pH = 6.9	

VP Test Reagents

1. *α-Naphthol, 5% color intensifier*
   α-Naphthol 5 g
   Absolute ethyl alcohol 100 mL
2. *Potassium hydroxide, 40%, oxidizing agent*
   Potassium hydroxide 40 g
   Distilled water to 100 mL

Quality Control

Positive and negative controls should be run after preparation of each new lot/batch of medium and reagent. Suggested controls are:

A. Positive Control: *Enterobacter aerogenes*

B. Negative Control: *Escherichia coli*

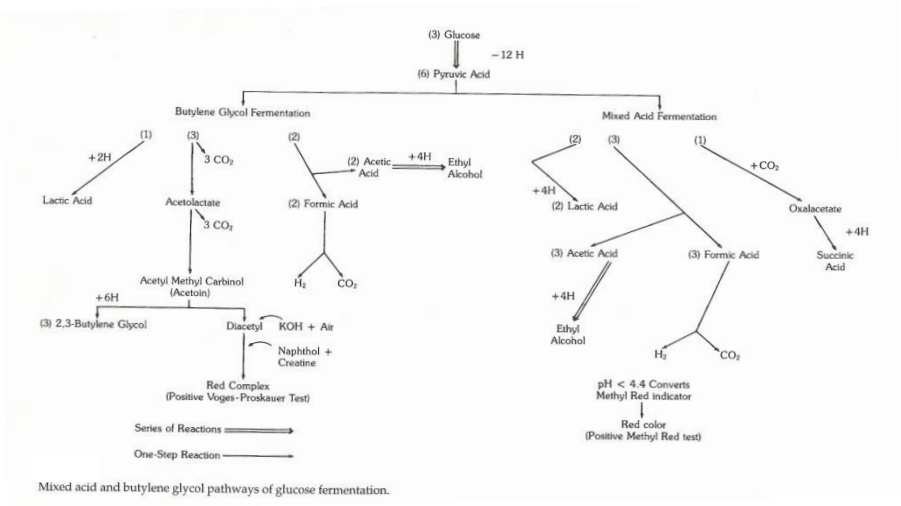
Procedure

A pure culture of the test organism should be inoculated in MR/VP broth. The test medium tube should be incubated for 24 hours at 35°C. After incubation, a 1 mL aliquot of broth should be tranferred to a clean test tube. Then, 0.6 mL of 5% α-naphthol should be added which should be followed by 0.2 mL of 40% KOH (strictly follow this sequence). The tube should be shaken gently to expose the medium to atmospheric oxygen and should be allowed to remain undisturbed for 10 to 15 minutes.

Results

Interpretation

If a red color develops in 15 minutes or more after addition of the reagents, the test results is positive. The formation of red color indicates the presence of diacetyl, the oxidation product of acetoin. The test should not be interpreted after standing for over 1 hour because negative Voges-Proskauer cultures may produce a copper-like color which may cause a false-positive interpretation.



Mixed acid and butylene glycol pathways of glucose fermentation.

Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*

Table.5.1.1. *Case problems.*

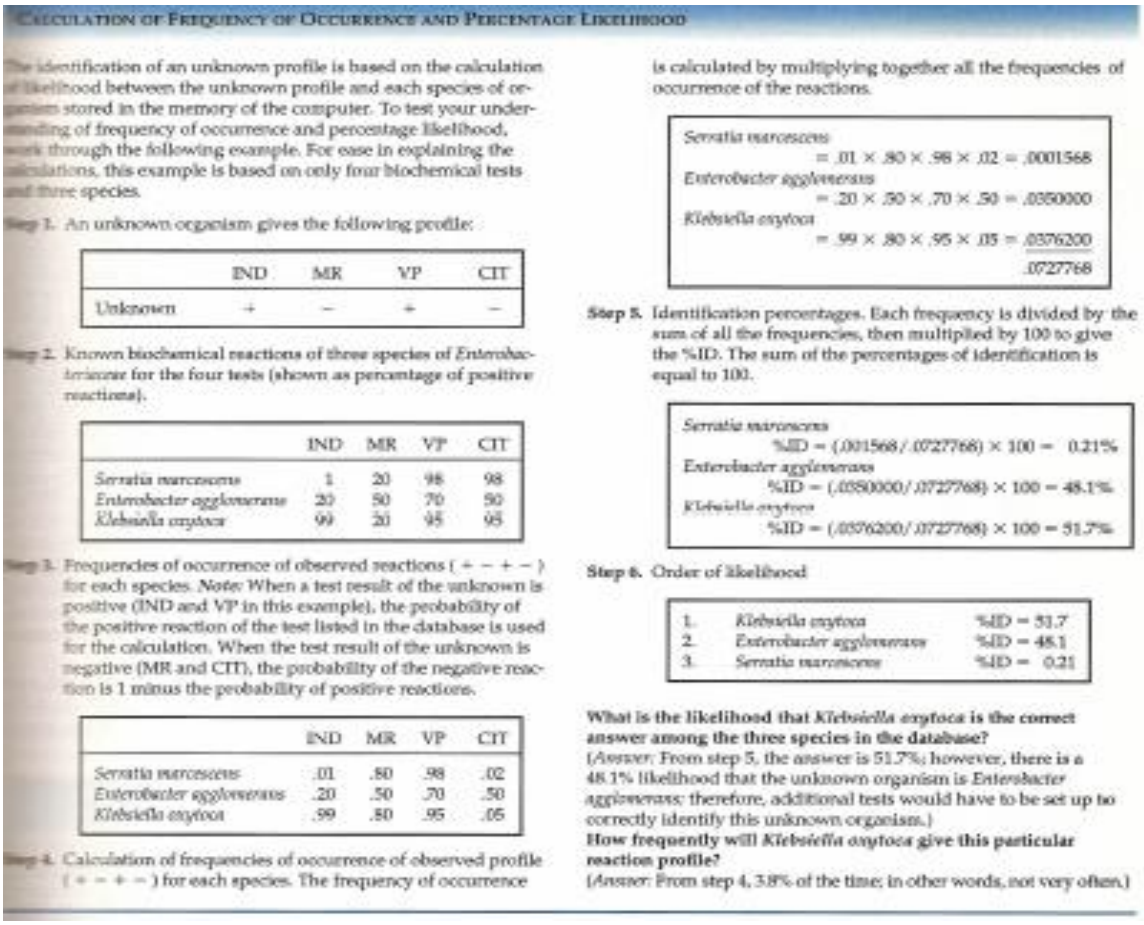
Identification of *Enterobacteriaceae*

Calculation of frequency of occurrence and percentage likelihood

		L/A	G/A	Gas	H_2S	MR	VP	IND	CIT	URE	MOT	*Taxon Name*	*L(%)*	Rank
B1	R	+	+	+	-	+	-	+	-	-	+			
	P1													
	P2													
	P3													
B2	R	-	+	-	-	+	-	-/+	-	-	-			
	P1													
	P2													
	P3													
B3	R	-	+	+	+	+	-	-	+	-	+			
	P1													
	P2													
	P3													
B4	R	+	+	+	+	+	-	-	+	+/-	+			
	P1													
	P2													
	P3													
B5	R	+	+	++	-	-	+	-	+	+	-			
	P1													
	P2													
	P3													
B6	R	-	+	+/-	+	+	-	+	-/+	++	+			
	P1													
	P2													
	P3													

B=Bacterium, P=Probability, L=Likelihood.

Example: (Reference: *Koneman E.W. 2005. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Lippincott Williams & Wilkins, Philadelphia.*)



CALCULATION OF FREQUENCY OF OCCURRENCE AND PERCENTAGE LIKELIHOOD

The identification of an unknown profile is based on the calculation of likelihood between the unknown profile and each species of organism stored in the memory of the computer. To test your understanding of frequency of occurrence and percentage likelihood, work through the following example. For ease in explaining the calculations, this example is based on only four biochemical tests and three species.

Step 1. An unknown organism gives the following profile:

	IND	MR	VP	CIT
Unknown	+	−	+	−

Step 2. Known biochemical reactions of three species of *Enterobacteriaceae* for the four tests (shown as percentage of positive reactions).

	IND	MR	VP	CIT
Serratia marcescens	1	20	98	98
Enterobacter agglomerans	20	50	70	50
Klebsiella oxytoca	99	20	95	95

Step 3. Frequencies of occurrence of observed reactions (+ − + −) for each species. *Note:* When a test result of the unknown is positive (IND and VP in this example), the probability of the positive reaction of the test listed in the database is used for the calculation. When the test result of the unknown is negative (MR and CIT), the probability of the negative reaction is 1 minus the probability of positive reactions.

	IND	MR	VP	CIT
Serratia marcescens	.01	.80	.98	.02
Enterobacter agglomerans	.20	.50	.70	.50
Klebsiella oxytoca	.99	.80	.95	.05

Step 4. Calculation of frequencies of occurrence of observed profile (+ − + −) for each species. The frequency of occurrence is calculated by multiplying together all the frequencies of occurrence of the reactions.

Serratia marcescens
= .01 × .80 × .98 × .02 = .0001568
Enterobacter agglomerans
= .20 × .50 × .70 × .50 = .0350000
Klebsiella oxytoca
= .99 × .80 × .95 × .05 = .0376200
.0727768

Step 5. Identification percentages. Each frequency is divided by the sum of all the frequencies, then multiplied by 100 to give the %ID. The sum of the percentages of identification is equal to 100.

Serratia marcescens
%ID = (.0001568 / .0727768) × 100 = 0.21%
Enterobacter agglomerans
%ID = (.0350000 / .0727768) × 100 = 48.1%
Klebsiella oxytoca
%ID = (.0376200 / .0727768) × 100 = 51.7%

Step 6. Order of likelihood

1.	*Klebsiella oxytoca*	%ID = 51.7
2.	*Enterobacter agglomerans*	%ID = 48.1
3.	*Serratia marcescens*	%ID = 0.21

What is the likelihood that *Klebsiella oxytoca* is the correct answer among the three species in the database?
(*Answer:* From step 5, the answer is 51.7%; however, there is a 48.1% likelihood that the unknown organism is *Enterobacter agglomerans*; therefore, additional tests would have to be set up to correctly identify this unknown organism.)
How frequently will *Klebsiella oxytoca* give this particular reaction profile?
(*Answer:* From step 4, 3.8% of the time; in other words, not very often.)

Table.5.1.2. *Reference frequencies.*

(%) Frequencies of Occurrence for Observed Biochemical Reactions in Bacterial Species											
Organism	**TSI**				**H_2S**	**MR**	**VP**	**IND**	**CIT**	**URE**	**MOT**
	G/Acid	**G/Gas**	**L/Acid (F)**	**S/Acid (F)**							
Escherichia coli	100	95	95	50	1	99	0	98	1	1	95
Shigella, O groups A, B, C	100	2	0	0	0	100	0	50	0	0	0
Salmonella	100	96	1	1	95	100	0	1	95	1	95
Citrobacter freundii	100	89	78	89	78	100	0	33	78	44	89
Klebsiella pneumoniae	100	97	98	99	0	10	98	0	98	95	0
Enterobacter aerogenes	100	89	78	89	78	100	0	35	78	44	89
Proteus mirabilis	100	96	2	15	98	97	50	2	65	98	95
Proteus vulgaris	100	85	2	97	95	95	0	98	15	95	95

What should you do?

Materials and methods:

Materials:

1. Demostrative oxidase test.
2. Demostrative TSI test.
3. Demonstrative indol and urease production, citrate utilization, motility, metyhl-red and Voges-Proskauer tests.
4. Case problems (Table.5.1.1.) for bacterial species identification.
5. Exemplary calculation of likelihood and ranking for bacterial species identification.
6. Reference frequencies (Table.5.1.2.) of occurrence for observed biochemical reactions in bacterial species.

Method:

7. Follow the instructions of the laboratory instructor.
8. Examine the demonstrative test results.
9. Examine the exemplary calculation of likelihood and ranking for bacterial species identification.
10. With reference to given frequencies (Table.5.1.2.), calculate the likelihoods; perform rankings; and identify binomial taxonomic names of bacterial species (i.e. hypothetical isolates with given phenotypical/biochemical characteristics) in the case problems (Table.5.1.1.).
11. Write your assessments in the "**discussion section**" of the laboratory report:
    a. *the materials, methods and results of bacterial species identification.*
    b. *discuss the reliability of the likelihood calculation and ranking approach in bacterial species identification.*

LABORATORY REPORT

DATE:

TITLE:

INTRODUCTION (Purpose of the study):

MATERIALS AND METHODS:

RESULTS:

DISCUSSION/CONCLUSION:

6.1. Microscopy and Culture Methods for Diagnosis of *Mycobacteria*

What should you know?

- Mycobacteria are widespread in environment and in animals
- Among mycobacteria, major human pathogens are *M. tuberculosis* and *M. leprae*
- Importance of other species (e.g. *M. avium* complex) is increasing in AIDS and other immunocompromised patients
- Aerobic rods with a Gram-positive cell wall structure, but stain with difficulty because of the long-chain fatty acids (mycolic acids) in the cell wall
- **Acid fastness (acid-fast staining bacteria; AFB) can be demonstrated by resistance to decolorization by mineral acid and alcohol (Ziehl-Neelsen stain)**
- **Mycobacteria grow more slowly than many other bacteria**
    - rapid growers (visible colonies formed within ca. 3-7 days)
    - slow growers (visible colonies formed only after ca. 2 weeks to 2 months' of incubation)
- **In laboratory identification, staining and microscopic examination of specimens for acid-fast rods is important because of the time required for culture results**
- **All species except *M. leprae* can be grown in artificial culture but they require complex media**
- **Nucleic acid methods are available for identification**
- **Expectorated sputum (productive cough), as a sample, can be a good representative of lower respiratory tract infections** (LRTIs) and should be cultured only if it is informative on the presence of inflammation **without excessive contamination with the pharyngeal microbiota.**
    - Therefore, **quality of a sputum specimen** should be assessed before accepting for bacterial culture. If sputum quality is not acceptable for culture, it should be rejected.
    - This is done by employing **a scoring system for sputum quality in gram-stained smears.**
    - Still, quality score would **not be valid for LRTIs caused by some infectious microorganisms such as, viral agents, *M. tuberculosis*, and other atypical pneumonia agents.**
- **A grading system for sputum specimens** should also be employed to **enumerate acid-fast bacilli in EZN stained smears.**
    - This procedure provides an **assessment of infectiousness of a patient infected with *M. tuberculosis*.**
    - As a mycobacterial culture may require long time for incubation up to 4 weeks to produce a positive result, **a same day result of stained smear** is important to initiate **empirical therapy and control disease spread** in population.
- Together with the employment of above mentioned scoring or grading systems in stained smears, **bacteriological culture of sputum specimen** provides the isolation of infectious agents causing LRTIs. **Isolation of the causative bacterial agent in sputum culture aids in making a definitive diagnosis for a LRTI.**

Acid-fast (Ziehl-Neelsen) staining method

Materials:

Acid-fast bacilli are surrounded by a waxy envelope and either heat or a detergent (Tergitol) is required to allow the stain to penetrate the capsule. Once stained, they are resistant to decolorization with acid-alcohol, whereas other bacteria are destained with the acid alcohol.

- Carbolfuchsin solution
  - Phenol crystals 2.5 mL
  - Alcohol,95% 5mL
  - Basic fuchsin 0.5 g
  - Distilled water 100 mL
- Acid alcohol solution, 3%
  - HCl, concentrated 3mL
  - Alcohol, 70% 100 mL
- Methylene blue solution
  - Methylene blue 0.5 g
  - Glacial acetic 0.5 mL
  - Distilled water 100 mL

Method

1. Place a 2 X 3-cm filter paper strip on the slide to help hold the stain on the slide and filter undissolved crystals.
2. Flood the paper strip with Ziehl-NeeIsen carbolfuchsin.
3. Using a Bunsen burner or electric staining rack, heat the slide slowly until steaming, but short of boiling.
4. Stain for 5 minutes. If the stain dries, add more stain without heating.
5. Carefully remove the paper slip from the slide using forceps.
6. Rinse the slide thoroughly with water.
7. Flood the smear with 3% acid-alcohol decolorizer for 2 minutes.
8. Rinse with water and drain excess water.
9. Flood the slide with methylene blue counterstain for 1 minute. Do not blot.
10. Examine the smear with a X 100 oil immersion objective.

Grading of AFB in EZN-stained smear

METHOD FOR REPORTING NUMBERS OF ACID-FAST BACILLI OBSERVED IN STAINED SMEARS			
NUMBER OF BACILLI OBSERVED	**CDC** METHOD REPORT		INTERPRETATION
0	Negative	(-)	May not be infectious
1-2/300 fields	Number seen'	(±)	Probably infectious
1-9/100 fields	Average no./100 fields	(1+)	Probably infectious
1-9/10 fields	Average no./10 fields	(2+)	Probably infectious
1-9 field	Average no. / field	(3+)	Probably very infectious
More than 9/field	More than 9/ field	(4+)	Probably very infectious

- Examination at X800 to x1000 is assumed. Magnifications less than X800 should be clearly stated. If a microscopist uses consistent procedure for smear examination, relative comparisons of multiple specimens should be easy for the clinician, regardless of magnification used. To equate numbers of bacilli observed at less than X800 with those seen under oil immersion, adjust counts as follows: for magnifications about x650, divide count by 2; near X450, divide by 4; near X250, divide by 10; e.g., if 8 bacilli per 10 fields were seen at X450, the count at X1000 would be equivalent to about 2/10 fields (8/4).

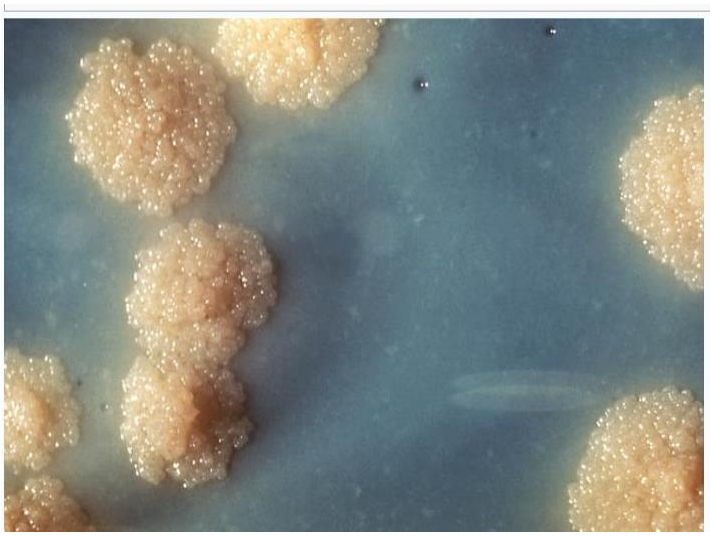
- Counts less than 3/3000 fields at x800 to x1000 are not considered positive; another specimen (or repeat smear of same specimen) should be processed if available.

(American Thoracic Society: Diagnostic standards and classification of tuberculosis and other mycobacterial diseases. Am Rev Respir Dis 123:343--358, 1981).

Culture media for mycobacteria

Mycobacteria;

- Grow slowly than most of the bacteria pathogenic for humans
- The rapid growing species form colonies in 2-3 days
- Whereas most pathogenic mycobacteria require 2-6 weeks of incubation in ***Lowenstein-Jensen medium***.
- This is reflected to the report of microbiology laboratory (6-8 weeks)



NON-SELECTIVE MYCOBACTERIAL ISOLATION MEDIA		
MEDIUM	**COMPONENTS**	**INHIBITORY AGENT**
Löwenstein-Jensen	Coagulated whole eggs, defined salts, glycerol, potato flour	Malachite green, 0.025g/100 mL
Petragnani	Coagulated whole eggs, egg yolks, whole milk, potato, potato flour, glycerol	Malachite green, 0.052g/100 mL
American Thoracic Society medium	Coagulated fresh egg yolks, potato flour, glycerol	Malachite green, 0.02g/100 mL
Middlebrook 7H10	Defined salts, vitamins, cofactors, oleic acid, albumin, catalase, glycerol, dextrose	Malachite green, 0.0025g/100 mL
Middlebrook 7H11	Defined salts, vitarnins, cofactors, oleic acid, albumin, catalase, glycerol, 0.1% casein hydrolysate	Malachite green, 0.0025g/100 mL

What should you do?

Method:

1. Follow the instructions of the laboratory instructor.
2. Examine the demonstrative ZN stained slides (x1000; immersion field).
3. Perform a scoring for a ZN stained specimen (examine 10 fields).
4. Examine the demonstrative culture media.
5. Write your assessments in the "**discussion section**" of the laboratory report:
    a. *discuss the importance of ZN staining.*
    b. *discuss the uses of interpretive results and reporting.*

LABORATORY REPORT

DATE:

TITLE:

INTRODUCTION (Purpose of the study):

MATERIALS AND METHODS:

RESULTS:

DISCUSSION/CONCLUSION:

7.1. Immunoassays in Diagnostic Microbiology

Agglutination
ELISA

What should you know?
Immunological methods are used to detect, identify, and quantitate antigen in clinical specimens, as well as to evaluate the antibody response to infectious agents and a person's history of exposure to an infectious agent.

The specificity of the antibody-antigen reaction and the sensitivity of many of the immunologic techniques make them powerful laboratory methods.

The same technique may be used to test different antigens and antibodies.

What should you do?
Follow the instructions of the laboratory instructor. Examine the demonstrative ELISA plate.

Materials:
1. Agglutination test kit.
2. *S. aureus* on agar plate.
3. Finalized ELISA plate and results.

Methods:
1. Perform an agglutination test as instructed.
2. Examine the colorimetric plate results as shown in demonstration.
3. Write your evaluation in the "**discussion section**":
    a. *the result of the agglutination test*
    b. *compare the colors of negative and positive test results in ELISA plate.*

LABORATORY REPORT

DATE:

TITLE:

INTRODUCTION (Purpose of the study):

MATERIALS AND METHODS:

RESULTS:

DISCUSSION/CONCLUSION:

8.1. Molecular Methods in Diagnostic Microbiology

Introduction:

Molecular Methods in Diagnostic Microbiology

The DNA (deoxyribonucleic acid), RNA (ribonucleic acid) of an infectious agent in a clinical sample can be used to help identify the agent. Significant changes continues to occur in diagnostic molecular microbiology. If selected, used and the results are interpreted properly, molecular techniques have the increasing advantage of sensitivity, specificity, safety and speed. The rapid growth of commercially available test kits and analyte-specific reagents has fascilitated the use of these methods in routine practice.

Target molecule

- **DNA**
- **RNA**

Molecular techniques can mainly classified into two main groups:

-Hybridizaton

-Nucleic acid amplification techniques

Hybridizaton:

Synthetic oligonucleotides of ≤ 50 nucleotides, labelled with radioisotopes, enzymes and chemiluminescent reporter molecules can be be used to detect pathogens in clinical specimens or identification of pathogens after isolation by culture. These oligonucleotides are called probes and they bind to the complementary nucleic acid sequences in the clinical sample with high degree of **specificity.** They lack sensitivity.

Nucleic acid amplification techniques:

These techniques have become the new gold standart for the laboratory diagnosis and monitoring of several infectious diseases and each they teir application is increasing. Therefore you should know the advantages and disadvantages and the important points to be considered in interpretation of the results.

Several amplification techniques are present. Polymerase chain reacton (PCR) is the best developed and most widely used nucleic acid amplification strategy. PCR uses the DNA replication 'machinery' of a cell to make multiple copies of a specific DNA sequence.

PCR is discovered in 1983 in California by Kary Mullis, published in a 1985 paper, sold by Cetus Corporation for $300 million, and Mullis won the 1993 Nobel Prize in Chemistry for his discovery.

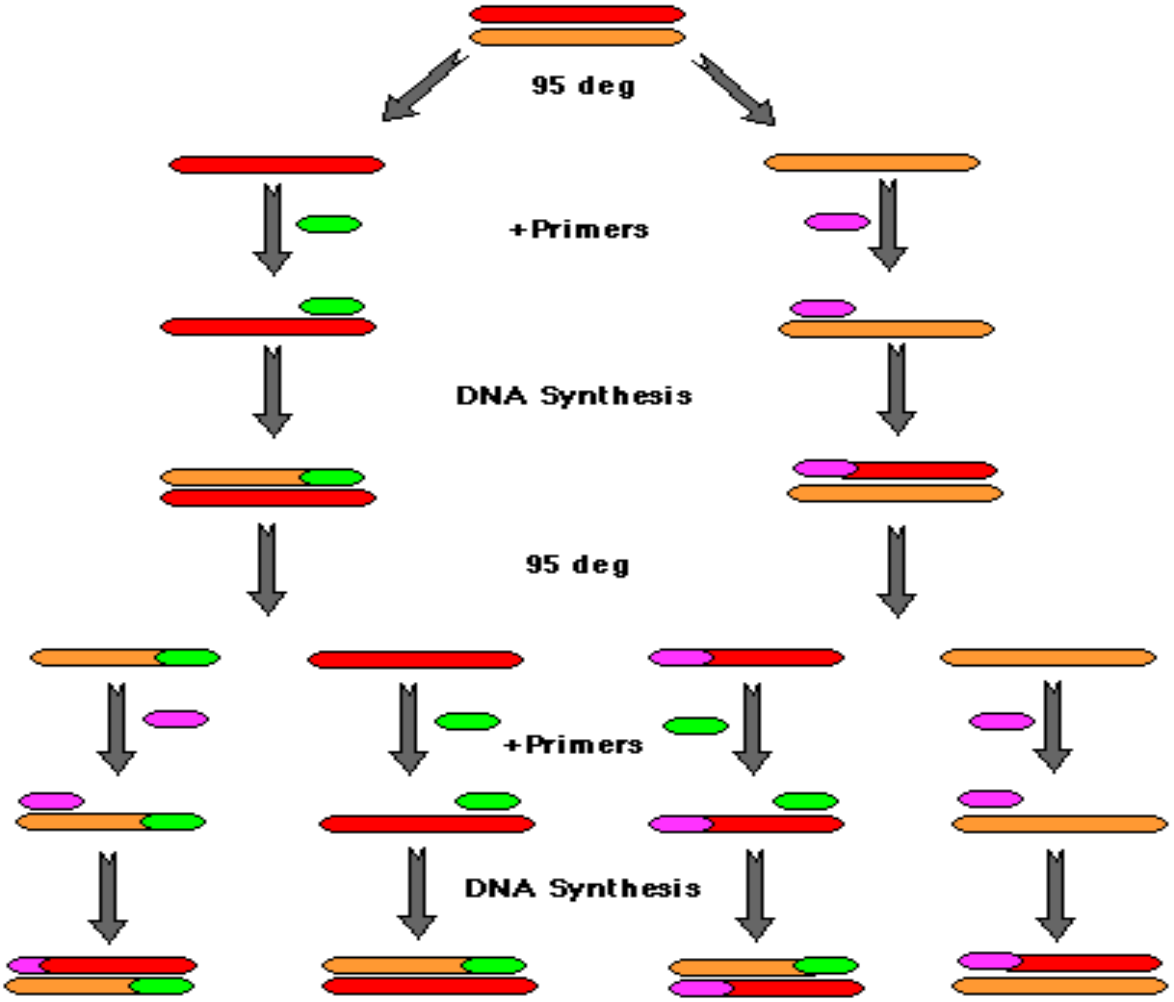
Nucleic acid amplification techniques consist of three major steps:

- Specimen processing: Nucleic acid extraction (mostly automated)
- Nucleic acid amplification
- Product detection

Requirements of PCR:

- ***Knowing parts of the target DNA sequence to be amplified***
- ***Two types of synthetic primers, complementary to the ends of the target sequence***
- ***Large amounts of the four DNA nucleotides***
- ***A heat stable DNA Polymerase***

How it works…



The three steps in PCR (polymerase chain reaction) are:

- denaturation,
- annealing of the primers to target, and
- extention.

Each extention product and the original target will serve as a template subsequent annealing and extention. At the end of each cycle the PCR product is theoretically doubled. The whole procedure is carried out in a programmable thermal cycler.

Postamplification detection step:

- Gel analysis
- Colorimetric microtitre plate system
- Target amplification and detection systems occur simultaneously in the same tube (Real- Time PCR)

Cumbersome nucleic acid extraction nucleic acid extraction methods have been replaced by simply applicable commercial kits.

Real-time PCR
The target amplification and detection steps occur simultaneously. This method require specific thermal cycler. These instruments have precise optical equipment that monitor the emission of fluorescent from the samples during amplification and also computer software which monitors the date during PCR amplification.

Multiplex PCR
Two or more primer sets designed for amplification of different targets are included in the same reaction mixture so that more than one target sequence can be coamplified in a single tube.

The main applications of NAT (nucleic acid technology/testing) in diagnostic molecular microbiology are:

1- We can identify the infectious agent in a clinical sample (Qualitative)
2- We can monitor the progression or the outcome of therapy (Quantitative)
3- We can detect antimicrobial resistance.

The interpretation of a "negative test" requires:

- assay sensitivity
- specimen quality
- nucleic acid extraction efficacy
- amplification efficency consideration.

Internal controls are included in reactions to control nucleic acid extraction efficacy and amplification efficency.

In this practice, the student:

1-will perform post amplification detection of already amplified DNA by gel electrophoresis
2-will observe automated real-time amplification application
3-will observe multiplex PCR amplification result interpretation

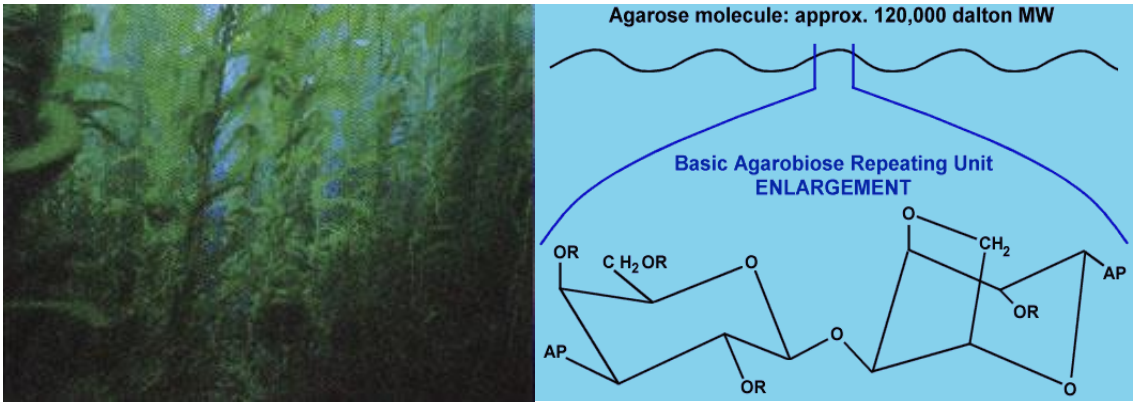
1-Genetic analysis: Genetic analysis of DNA by electrophoresis

- Electrophoresis is the migration of charged molecules in a porous matrix (gel) in response to an electric field.
- The rate of migration of the molecules depend on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving.
- As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the size and other features of molecules.

Gel Matrices Used in Electrophoresis

- The most commonly used support matrices - agarose and polyacrylamide - provide a means of separating molecules by size, in that they are porous gels.
- A porous gel may act as a sieve by retarding, or in some cases completely obstructing, the movement of large macromolecules while allowing smaller molecules to migrate freely.
- Because dilute agarose gels are generally more rigid and easy to handle than polyacrylamide of the same concentration, agarose is used to separate larger macromolecules such as nucleic acids, large proteins and protein complexes.
- Polyacrylamide, which is easy to handle and to make at higher concentrations, is used to separate most proteins and small oligonucleotides that require a small gel pore size for retardation.

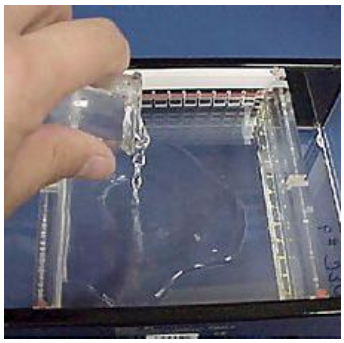
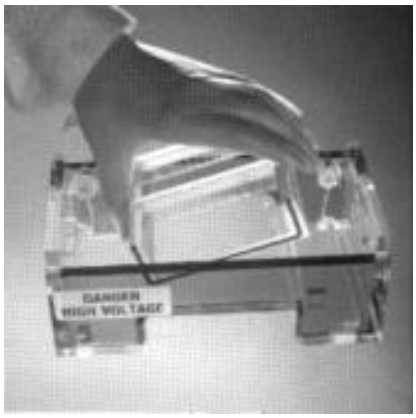
Agarose Gel Electrophoresis

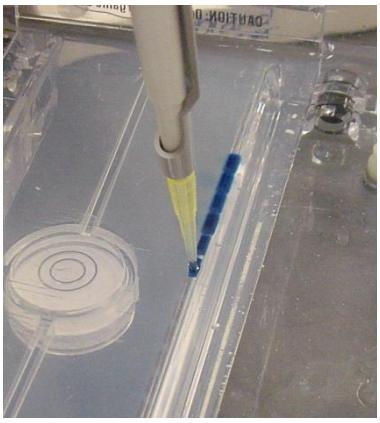
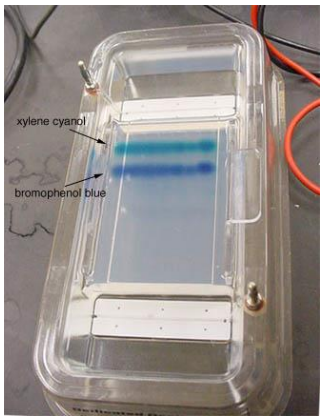


- Agarose comes from a family of polysaccharides called agars that are obtained from algae such as seaweed. They range from neutral agar to highly charged carrageenan. Biological function in seaweed & algae is antidessication at low tide, and also provide some mechanical support so that cells do not collapse.
- They are used extensively in food industry as ingredient stabiliser e.g. in ice cream, instant whips and dessert Jelly, culture media and electrophoresis gels.
- Structurally, agarose is a galactan (galactose polymer) in which the linkages alternate 1-4, 1-3, 1-4, 1-3. This arrangement allows two chains to join together and adopt a left handed double helix. The two chains wrap together so tightly that gaps are closed, and water is trapped inside the helix.

- Agarose gel electrophoresis is the easiest and commonest way of separating and analyzing DNA and RNA. Proteins are too small to be separated by agarose gel.
- The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band belonging to a certain size DNA molecules.
- The DNA is visualised in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. Other fluorescent dyes that bind to DNA can also be used for staining.



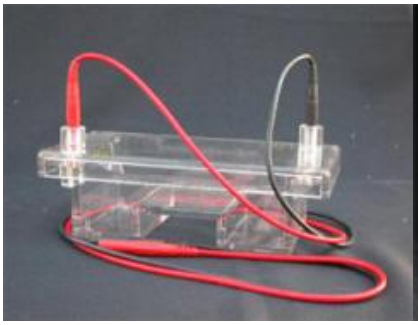



Assay:

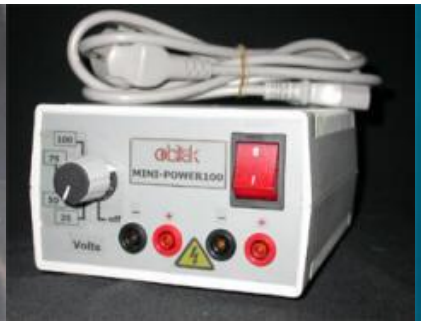
In this practice you will perform electrophoresis using the instruments and material provided for agarose gel electrophoresis.

Provided materials:

1- Electrophoresis instrument
2- Power supply
3- Ready to use agarose gel
4- Ready to use electrophoresis buffer
5- Prestained visible samples
6- Gel loading buffer.



Electrophoresis instrument



Power Supply

Prestained samples

Application:

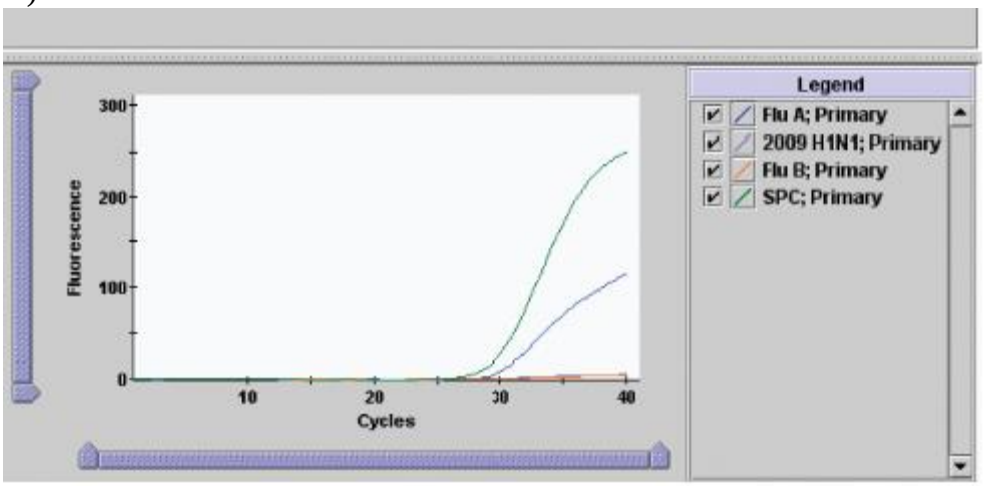
Each group will use one electrophoresis instrument; two groups will share one power supply.

1- Wear gloves.
2- Open carefully the package of agarose gel whitout breaking
3- Place the gel into the electrophoresis instrument. . It is very important that you should place the side of the gel where there are wells toward the black electrode side. The black electrode is the negative and the red electrode is the positive electrode and since the DNA is negatively charged the molecules will move from negative (black) to positive (red).
4- Pour the electrophoresis buffer into the electrophoresis instrument so that the whole gel will be covered and the level of the buffer be 2-3mm above the gel.
5- Cut a small peace of parafilm and place it on your desk.
6- Using automatic pipettor and disposable tip take 5µl samples and place on the parafilm as small drops. (Ask your instructor how to adjust and use the automatic pipettor.)
7- From each prestained sample take 10µl and mix with the loading buffer drops on the parafilm.
8- Using the automatic pipettor take the mixed samples one by one and load them into the wells.
9- Close the cap and be sure that the black electrode is on the side where you loaded the samples.
10- Connect the black wire of the instrument to black (negative) hole of the power supply and the red wire to red (postive) hole of the power supply.
11- Connect the power supply to the main power line.
12- Turn on the power supply and adjust to 100 Volts.
13- Observe the separation of stained molecules.

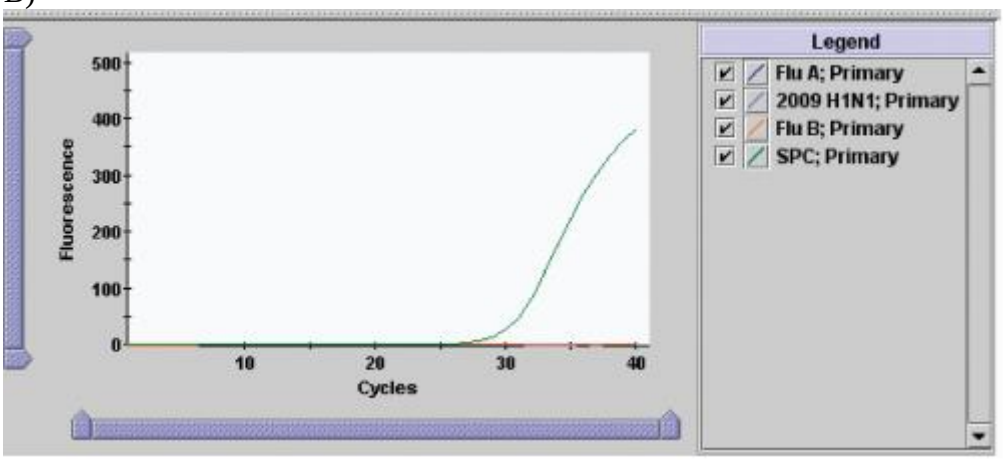
Discussion:

1-What is the disadvantage of amplification and detection by gel electrophoresis?
2-Please interpret the automated real-time PCR results shown below.

A)



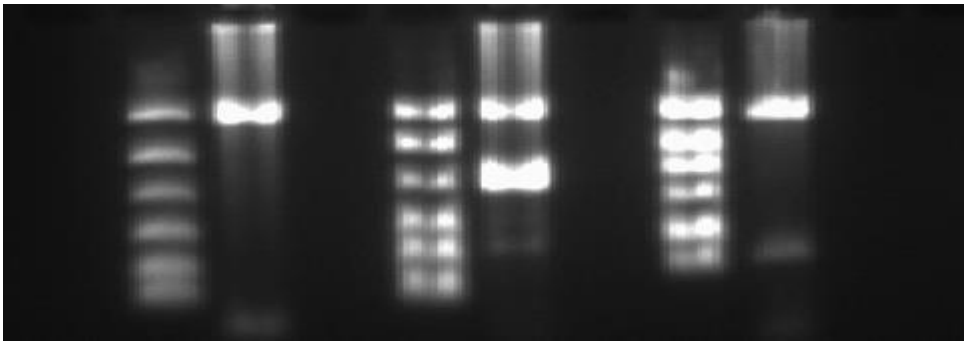
B)



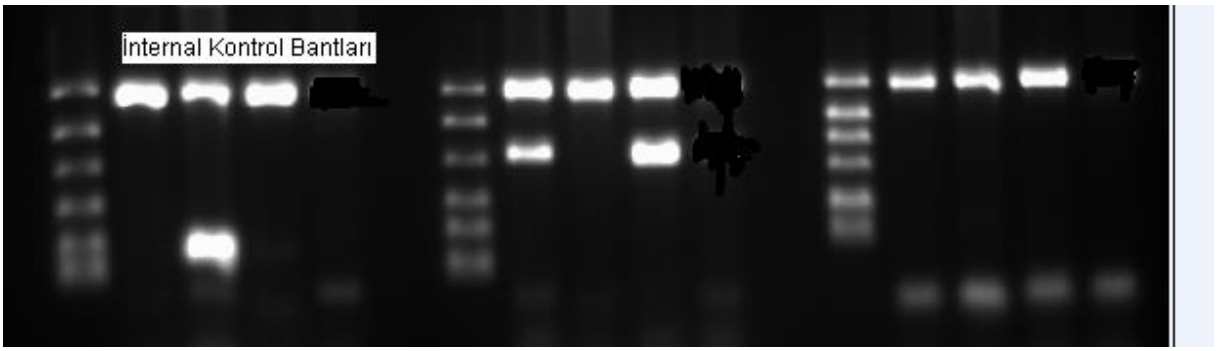
3-What is the advantage of real-time PCR?

4-What is the advantage of multiplex PCR result?

5-Interpret the result of multiplex PCR result shown below.



6- Interpret the result of multiplex PCR result shown below.



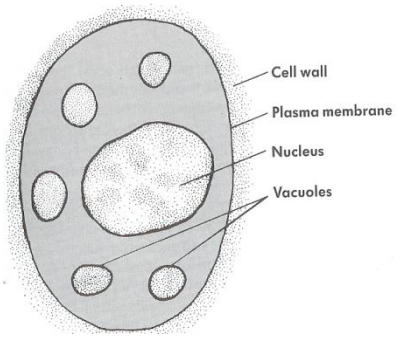
9.1. Mycology

The fungi (singular: fungus) are a diverse group of eucaryotic organisms. Some, such as the mushrooms have tissuelike differentiation and exist in nature as multicellular masses that are large enough to be seen with unaided eye. Other fungi, sucy as the bread and fruit moulds are composed of filamentous cells that are microscopic size or can be also seen with an unaided eye. Even the largest form lives at least one part of its life cycle in a single-celled form called **a spore**. But these funal spores are not as resistant to environmental stresses as are bacterial spores.

Almost all fungi have cell walls that contain chitin, cellulose, or hemicellulose as the structurally rigid component. (bacteria contain only peptidogylcan as the structurally rigid component)

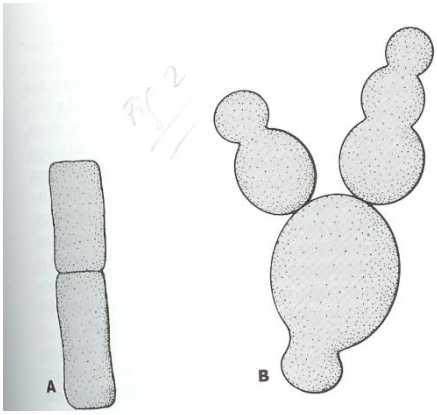
All fungi are strict chemoorganotrophs (like all protozoa and most bacteria); that is they require reduced organic compounds as a source of energy and carbon for growth. . Therefore all fungi are nonphotosynthetic and grow well in total darkness.

Morphology:
The " body" of a fungus is called a thallus (plural=thalli), can vary from a single cell to a complex shape that is composed of masses of cells and spore.
If the thallus is a single cell under most or all environmental conditions, the fungus is called a yeast (figure 1).

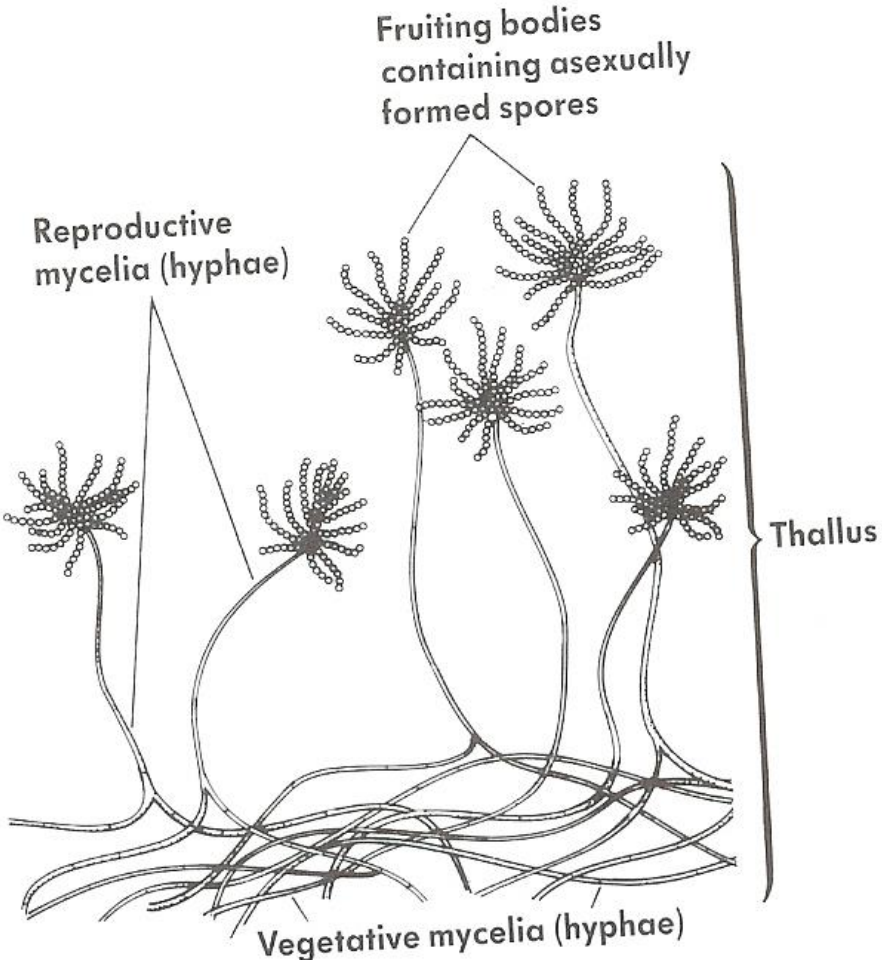


Size: Most yeasts are about 5-10 times larger than the length of average E.coli cell. They are commonly egg –shaped.

Yeasts can reproduce either:
1) asexually by transverse, binary fission or by forming buds or
2)sexually, conjugating wiht another cell and forming spores. (figure 2).



If thallus is composed of very long filaments under most or all environmental conditions, then the fungus is called a **mold** (figure 3).



The long filaments are called **mycelia** (singular: mycelium), but they may also be called **hyphae** (singular: hypha). The mycelia are often branched. If a branch terminates in a group of spores, this filament is called a *reproductive mycelium* to differentiate it from the branched and other interwoven begetative mycelia from which it arose.
Moulds can reproduce either asexually, by forming spores at ath the end of reproductive mycelia, or sexually wen adjacent cells conjugate to form spores.

Some fungi (called: dimorphic) can exist in either a singel-celled yeast form or a filamentous form.
The temperature at which most fungi grow best is between 20 and 30 C. They prefer to grow in a medium with and acid pH (pH 6 is optimum for the growth of most fungi).

9.1.1.Laboratory diagnosis of Yeasts

Yeasts are the most common cause of human fungal infections and, being opportunistics, their severity is greatly enhanced in the presence of predisposing factors: such as: immunosuppressive drug therapy, diabetes, prosthetic devices and drug abuse. Isolation of a yeast is not in itself diagnostic yeasts occur as part of the commensal flora on the surface of the body and in the gastrointestinal tract. Except in aseptically collected specimens of normally sterile material, interpretation of the mycological results is necessary. The clinical signs and symptoms and existence of predisposing factors will be helpgul in determining the significance of a yeast isolate.

Direct examination:

Microscopical examination: Give an erly indication of the presence of yeasts. Especially observing them in normally sterile tissue or fluids is significant (provided the specimens have been collected aseptically) .
The contaminated material such as faeces and sputum the observation is much less conclusive.

Tissue: A 1 mm thick section of the tissue is transferred to a drop of 20% potassium hydroxide (KOH) on a microscope slide. Allow to stand for a few minutes, place a coverslip on top and tap gently with rod. The unstained preparation may be viewed by light microscope but is is usually beneficial to examine under phase contrast or darkground microscopy. Alternatively it is procelssed as above but a drop of 0.1% Calcoflour white in 0.05% Evans blue to the 20% KOH is added and viewed under a fluorescent microscope.

Swabs. The smear is prepared, heat fixed and stained by Gram stain. Budding yeast cell and yeast hpyphae are seen as gram positive.

Cerebrospinal fluid: It should be concentrated by centrifugation before making any attempt to carry out microspcopy. Retain the supernatant for cryptococcal antigen testing. The deposite (precipitate) should be gram stained: only if budding cells are found shoud a further preparation be made to examine for capsule formation.
To a drop of the resuspended sediment a drop of india ink is added. Capsules appear as lager clear areas surrounding the yeast cell. When present; capsules are presumptive evidence of *Cryptococus neoformans*.

Urine: Concentration by centrifugation is necessary. A smear should be prepared from the sediment and Gram stained. Care should be taken during collection to minimize contamintion especially form the vagina in women.

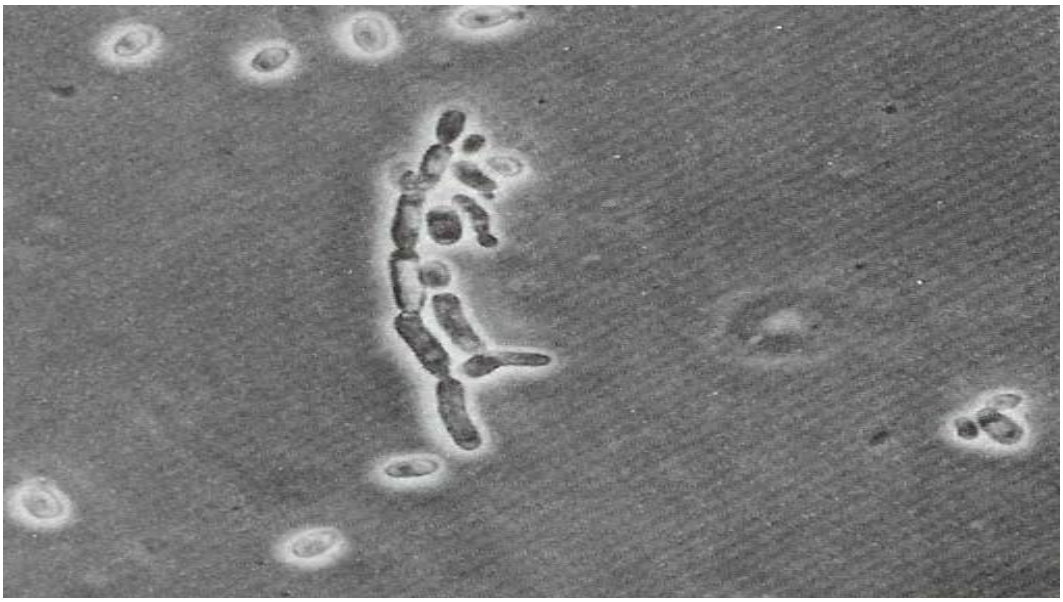
Culturing

Saboraud's dextrose medium is used.
Yeast colonies that are pathogenic to humans appear as opaque, pale colored, often with a sweet smell of bakeries.
Yeast colonies may be confirmed microscopically by emulsifying a portion of the colony in sterile water and staining

Germ tube test:

About 90% of yeasts isolated from human material are Candida albicans and a rapid method of identification is available. They have ability to produce a pseudo-germ tube in serum.

For this test take a single colony with a loop and emulsify the yeast cells in 0.5ml of horse or other serum in a small test tube. Incubate at 37 C for 2-4 h. Prolonged incubation is not recommended as mycelium production can obscure the germ tubes which are seen as extensions of a typical yeast cell and give a drumstick appearance.



Urease test:
Cryptococcus can be differentiated from Torulopsis by its ability to produce urease.

Sugar assimilation test (auxanogram):
Assimilation tests are commonly used for species identification of yeasts. The method consists essentially of growing a yeast on a basal carbohydrate-free medium supplemented with the test sugar.

Serodiagnosis

Serological tests are of little practical use in the diagnosis of systemic candidiosis. Rising titers of precipitins or agglutinins may provide a valid index but all too often ther is insufficient time to allow a prognostic diagnosis.

A latex agglutination test for cryptococcal antigen in CSF (Cerebro Spinal Fluid) and serum is commercially available and is of value in the differential diagnosis of meningitis. And also provided a means of monitoring therapy and can, in the case of AIDS patients.

The purposes of this laboratory exercise are:

- to introduce you to the size of a typical yeast cell (so that you will recognize it as a yeast when you see it again),
- to show you one method of asexeual reproduction accomplished by the yeasts by budding and
- to evaluate germ tube formation of *Candida albicans.*

Materials:

1. Cultures:
   1.1. Candida albicans
   1.2. Staphylococcus aureus
2. Media:
   2.1. Saboraud dextrose agar
3. Serum inoculated with *Candida albicans* for evaluation of germ tube formation
4. Gram staining set
5. Inoculation loop
6. Disinfectant container

Method:

1. Examine the colony morphology and the odor of the *Candida albicans* and compare with *S.aureus* colonies
2. Divide a glass slide into 2 and prepare a heat fixed slide of both *Candida albicans* and *S. aureus.*
3. Gram stain
4. Observe them under high magnification (X100) and draw your findings on your results section of your report.
   4.1. Record the budding yeast cells
   4.2. Since both of them will be stained and observed as Gram positive cocci therefore what is the difference between them? (Compare their size and shape and budding)
5. Prepare a wet mount of *Candida* inoculated serum.

Laboratory report:

1. Observe **germ tube formation** and draw your findings on your "**results section**" of your report.

2. In your discussion section:
   2.1.1. Compare the size difference of *Candida albicans* and *S. aureus*.
   2.1.2. Which cells contain buds? *C. albicans* or *S. aureus*?
   2.1.3. How many budding cells did you count per high magnification field?

Wet mount preparation:

1. Flame the inoculating loop to redness.
2. Remove the plug from the culture, and briefly flame the tip of the tube.
3. Remove two loopfuls of the culture and place them in the center of a clean glass microscope slide.
4. Reflame the tip of the culture tube and replace the plug.
5. Reflame the inoculating loop before putting it down.
6. Gently place a cover slip on the droplet.
7. Examine under low power and oil immersion

For Gram staining method:

a)Put a drop of saline on to the clean slide.

b)Heat an inoculating loop to redness. Cool it at the edge of the agar plate.

c)Take a colony from the agar plate with the inoculating loop and spread it next to the saline droplet.

d) Mix the colony with saline droplet until the mixture is smooth in texture.

e) Allow it to dry

f)Reflame the inoculating loop before putting it down.

g)With the sample side up pass the dried slide through the flame of the Bunsen burner several times. Do not over heat. (This proceudure will coagulate the protoplasm of the microorganism, causing them to stick -"be fixed"- to the slide).

Gram Staining Technique:

1st step: the fixed smear is stained with crystal violet for 30 sec
Wash it with Water
2nd step: iodine solution is applied. = for 30 sec
This step increases the interaction of dye with the cell wall.
Wash it with Water
3rd step Decolorizing with ethanol of acetone= for 30 sec
Wash it with water
Gram positive: retain the crystal violet, gram negative lose it.
4th step counterstaining with safranin= for 30 sec
Wash it with water
Gram negative stain pink to red, gram positive stays purple or dark blue.

9.1.2.Identification of Moulds

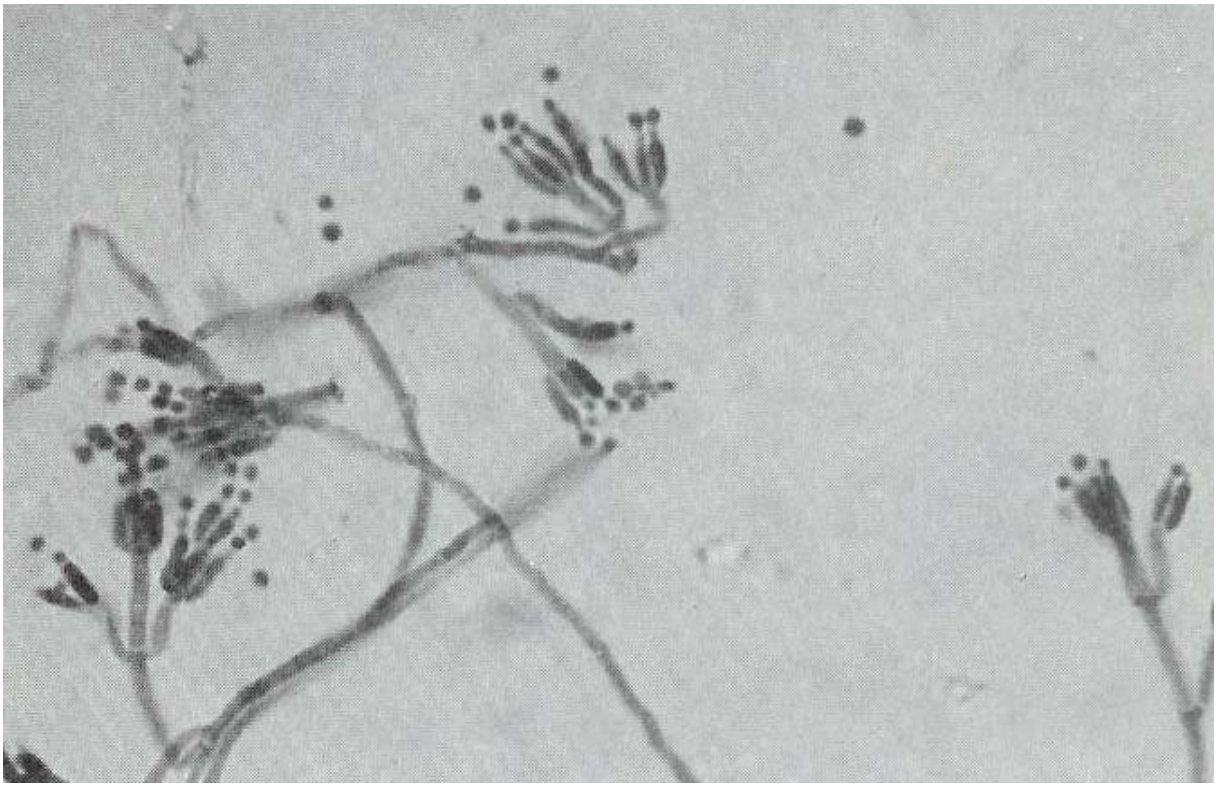
Purpose of this laboratory work is to:

- evaluate moulds by observing them, first macroscopically.
- second microscopically, for microscopic evaluation a different staining technique will be used: Lactophenol cotton blue stain.

Materials:

1. Culture
    a. Various mould cultures on Saboraurd Dextrose agar
2. Lactophenol cotton blue stain
3. Hooked inoculation needle
4. Disinfectant container

Figure.1. *Penicillium* hypae with Lactophenol cotton blue staining.



Method:

1. Observe the agar plate culture and record the following observations in your "**results section**":
    a. The color and textural appearance of the top of cultures as viewed with the unaided eye
    b. The color and general appearance of the bottom of culture as viewed with the unadided eye.
2. Take your hooked inoculation needle and sterilize it with your bunsen burner.
3. Cool it at the edge of the agar
4. Cut a piece of the colony along with the agar with your hooked loop and place it on a clean glass slide.
5. Put a drop of Lactophenol cotton blue stain on it and cover it with a cover slip.
6. With your handle section of your inoculation needle gently press the cover slip.
7. Observe under X10 and X40 magnification.
8. Draw your findings in your "**results section**".
9. Discuss the possible answers to following questions in "**discussion section**".
    a. What was the color of the colonies that you had observed?
    b. Describe the appearance of mycelia. Were they septate or nonseptate?
    c. What type of inoculation loop did you used for this purpose?
    d. What type of staining technique did you used? (Differential or simple staining?)

10.1. Parasitology-I

- **Idenfication of blood parasites**
- ***Preparation of thin and thick blood smears for the diagnosis of malaria***

Normal peripheral blood:

The usual diagnostic approach to blood disorders is blood counting and blood film examination. For the identification and study of human blood cells from peripheral smears, "Wright /Giemsa or May-Grünwald stain is generally used. It is a mixture of methylene blue, methylene azure and the eosinate blue and eosinate azure. The azures act as bases and stain the basophilic elements of the blood cells blue, while the eosins act as acids and stain the acidophilic structures red. Therefore with these types of stains the structures in blood cells have characteristic colors, and each cell type is readily identifiable. Mild variation in shape (poikilocytosis) and size (anisocytosis) is seen. Platelets appear as small bluish-purple discs. During blood film examination, the individual types of white blood cells are enumerated; this is referred to as the **differential count.**

In peripheral blood smear there are 3 main classes of cells:

Red blood cells : Erythrocytes

White blood cells are placed into 2 groups

- The granulocytes: have cytoplasmic granules classified as

Neutrophils (polymorphonucleated leucocytes=PNL)

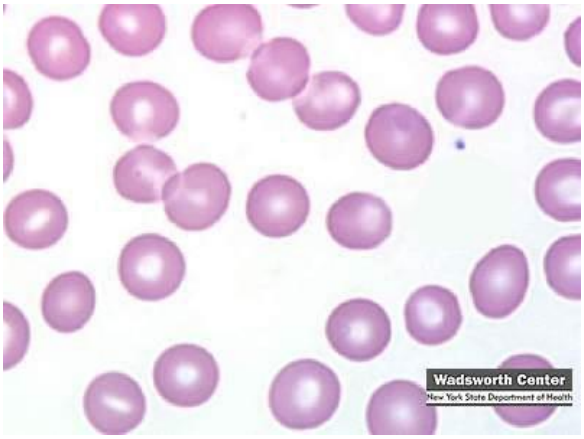
Eosinophils

Basophils

- The agranulocytes: lack the cytoplasmic granules:

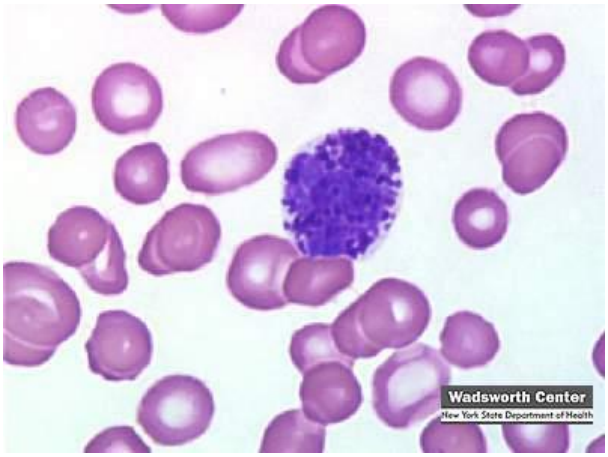
Lymphocytes

Monocytes



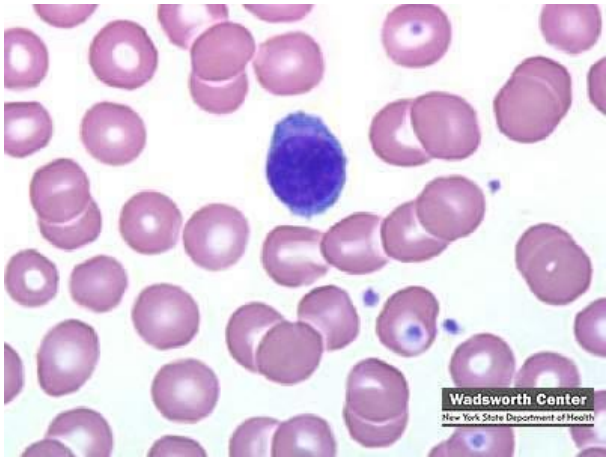
Erythrocytes (red blood cells)

The mature red blood cell (rbc) consists primarily of hemoglobin (about 90%). The membrane is composed of lipids and proteins. In addition, there are numerous enzymes present which are necessary for oxygen transport and cell viability. The main function of the red cell is to carry oxygen to the tissues and return carbon dioxide from the tissues to the lungs. The protein hemoglobin is responsible for most of this exchange. Normal red blood cells are round, have a small area of central pallor, and show only a slight variation in size. A normal red cell is 6-8 µm in diameter. As the relative amount of hemoglobin in the red cell decreases or increases, the area of central pallor will decrease or increase accordingly.



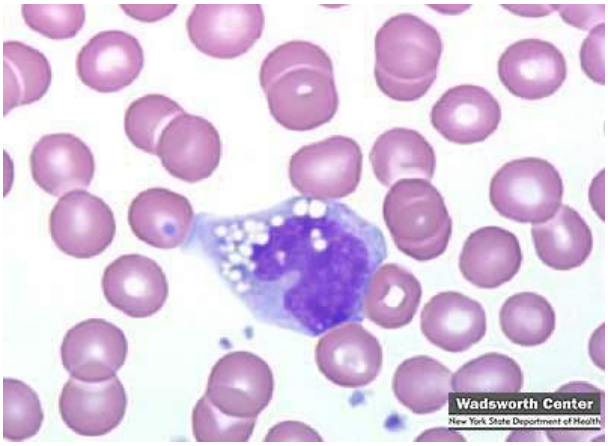
Basophil

Basophils are granulocytes that contain purple-blue granules that contain heparin and vasoactive compounds. They comprise approximately 0.5% of the total leukocyte count. Basophils participate in immediate hypersensitivity reactions, such as allergic reactions to wasp stings, and are also involved in some delayed hypersensitivity reactions. Basophils are the smallest circulating granulocytes, averaging 10 to 15 µm in diameter. The nucleus to cytoplasm ratio is about 1:1, and the nucleus is often unsegmented or bilobed, rarely with three or four lobes. The chromatin pattern is coarse and patchy, staining a deep blue to reddish-purple. The cytoplasm is a homogenous pale blue, but this is often obscured by the large dark granules.



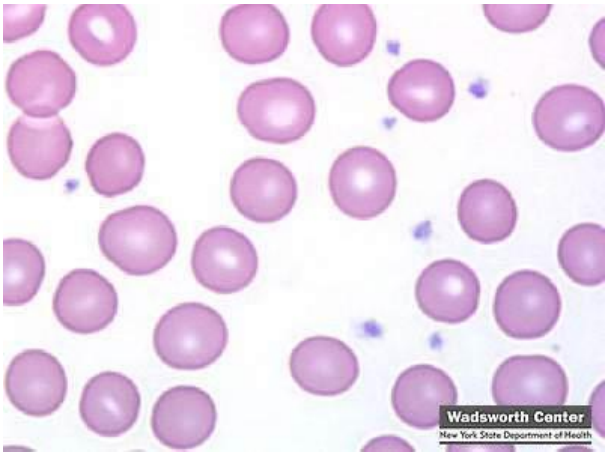
Lymphocyte

Lymphocytes in the peripheral blood have been described on the basis of size and cytoplasmic granularity. Small lymphocytes are the most common, ranging in size from 6 to 10 µm. The nucleus is usually round or slightly oval, occasionally showing a small indentation due to the adjacent centrosome. Except in the smallest cells, the nucleus is about 7 µm in diameter, a size that has been convenient for estimating the size of the surrounding erythrocytes. Nuclear chromatin stains a dark reddish-purple to blue with large dark patches of condensed chromatin. The nuclear cytoplasm ratio is 5:1 to 3:1, and the cytoplasm is often seen only as a peripheral ring around part of the nucleus.



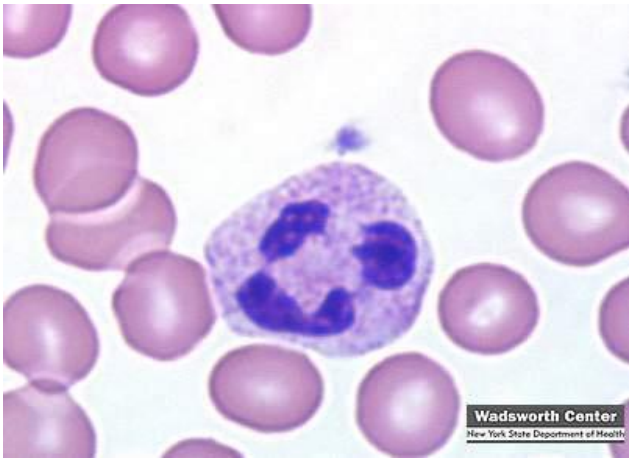
Monocyte

Monocytes are large mononuclear phagocytes of the peripheral blood. They are the immature stage of the macrophage. Monocytes vary considerably, ranging in size from10 to 30 µm in diameter. The nucleus to cytoplasm ratio ranges from 2:1 to 1:1. The nucleus is often band shaped (horseshoe), or reniform (kindey-shaped). It may fold over on top of itself, thus showing brainlike convolutions. No nucleoli are visible. The chromatin pattern is fine, and arranged in skein-like strands. The cytoplasm is abundant and blue gray with many fine azurophilic granules, giving a ground glass appearance. Vacuoles may be present



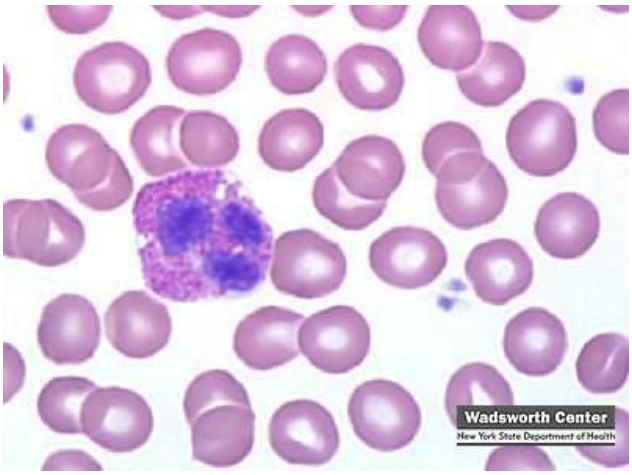
Platelets

Platelets are the cytoplasmic fragments of megakaryocytes, circulating as small discs in the peripheral blood. They are responsible for hemostasis (the stoppage of bleeding) and maintaining the endothelial lining of the blood vessels. During hemostasis, platelets clump together and adhere to the injured vessel in this area to form a plug and further inhibit bleeding. Platelets average 1 to 4 µm in diameter. The cytoplasm stains light blue to purple, and is very granular. There is no nucleus present. Normal blood concentrations range from 130,000 to 450,000/µL.



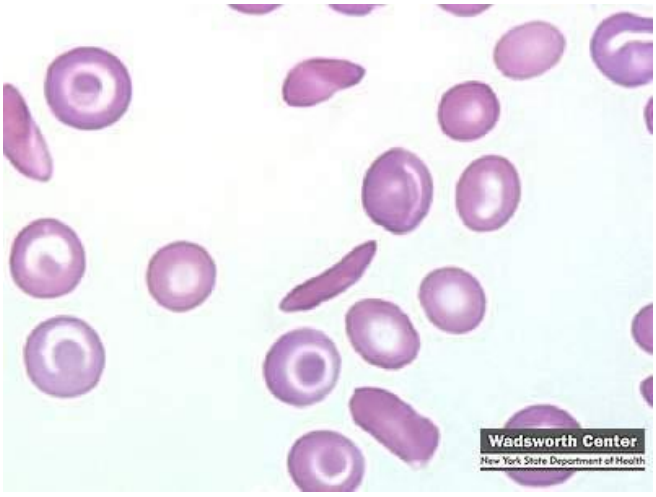
Segmented neutrophil (seg)

Segmented neutrophils (polymorphonuclear leukocytes, or segs) are the mature phagocytes that migrate through tissues to destroy microbes and respond to inflammatory stimuli. Segmented neutrophils comprise 40-75 % of the peripheral leukocytes. They are usually 9 to 16 µm in diameter. The nuclear lobes, normally numbering from 2 to 5, may be spread out so that the connecting filaments are clearly visible, or the lobes may overlap or twist. The chromatin pattern is coarse and clumped. The cytoplasm is abundant with a few nonspecific granules and a full complement of rose-violet specific granules.



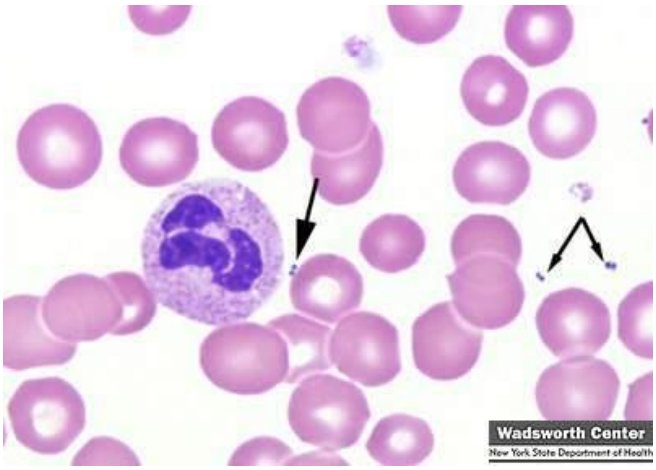
Eosinophil

Eosinophils are the mature granulocytes that respond to parasitic infections and allergic conditions. Eosinophils comprise about 1 to 4% of the peripheral leukocytes. They are usually 9 to 15 µm in diameter. Granules stain a bright reddish-orange with Wright's or Giemsa stains. The nucleus contains one to three lobes. The chromatin pattern is coarse and clumped. The cytoplasm is abundant with a full complement of bright reddish-orange specific granules.



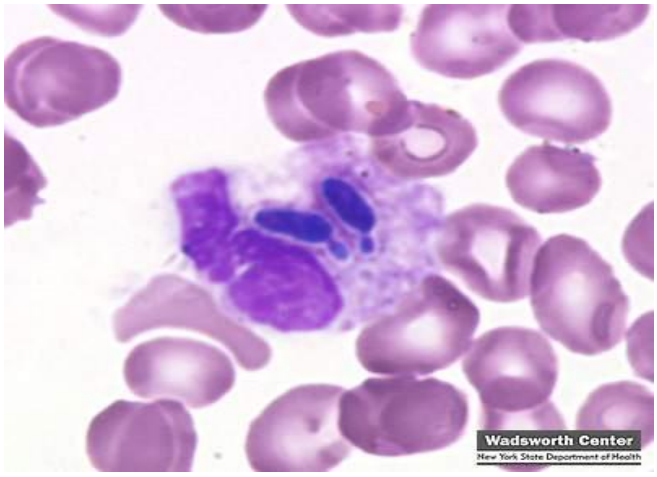
Sickle cells

Sickle cells are red blood cells that have become crescent shaped. When a person with sickle cell anemia is exposed to dehydration, infection, or low oxygen supply, their fragile red blood cells form liquid crystals and assume a crescent shape causing red cell destruction and thickening of the blood. Since the life span of the red blood cell is shortened, there is a temporary depression of red cell production in the bone marrow, and a subsequent fall in hemoglobin (and therefore the resultant anemia).



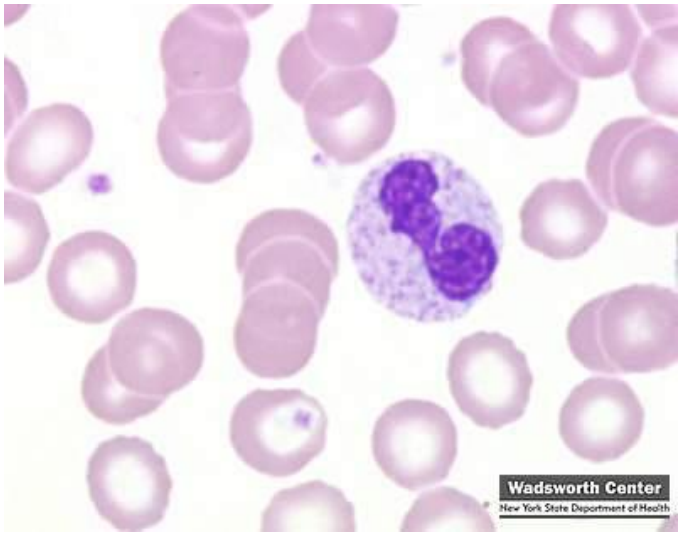
Bacteria

The image below illustrates a neutrophil with bacteria (Gram positive cocci). The major function of the neutrophil is to stop or retard the action of foreign matter or infectious agents. The neutrophil accomplishes this by moving to the area of inflammation or infection, phagocytizing (ingesting) the foreign material, and killing and digesting the material. Bacterial sepsis can result in a leukemoid reaction, involving white cell counts of 100,000 X 10^6/L (normal WBC=4,500 to 11,000 X 10^6/L), the presence of myelocytes, and the appearance of toxic granulation. This image was taken from a patient with *Streptococcus pnemoniae* infection.



Yeast

The image below illustrates a neutrophil with intracellular yeast. The major function of the neutrophil is to stop or retard the action of foreign matter or infectious agents. The neutrophil accomplishes this by moving to the area of inflammation or infection, phagocytizing (ingesting) the foreign material (in this case, the yeast), and killing and digesting the material.



Toxic granulation

Toxic granulation appears as dark blue-black granules in the cytoplasm of neutrophils. These granules are thought to be primary granules. Artifactual heavy granulation caused by poor staining is seen evenly spread throughout each cell and in all granulocytes, whereas toxic granulation is unevenly spread throughout the cytoplasm of certain cells. Large amounts of toxic granulation can give the neutrophil a bluish appearance.Toxic granulation is a stress response to acute infections, burns, and drug poisoning.

Materials:

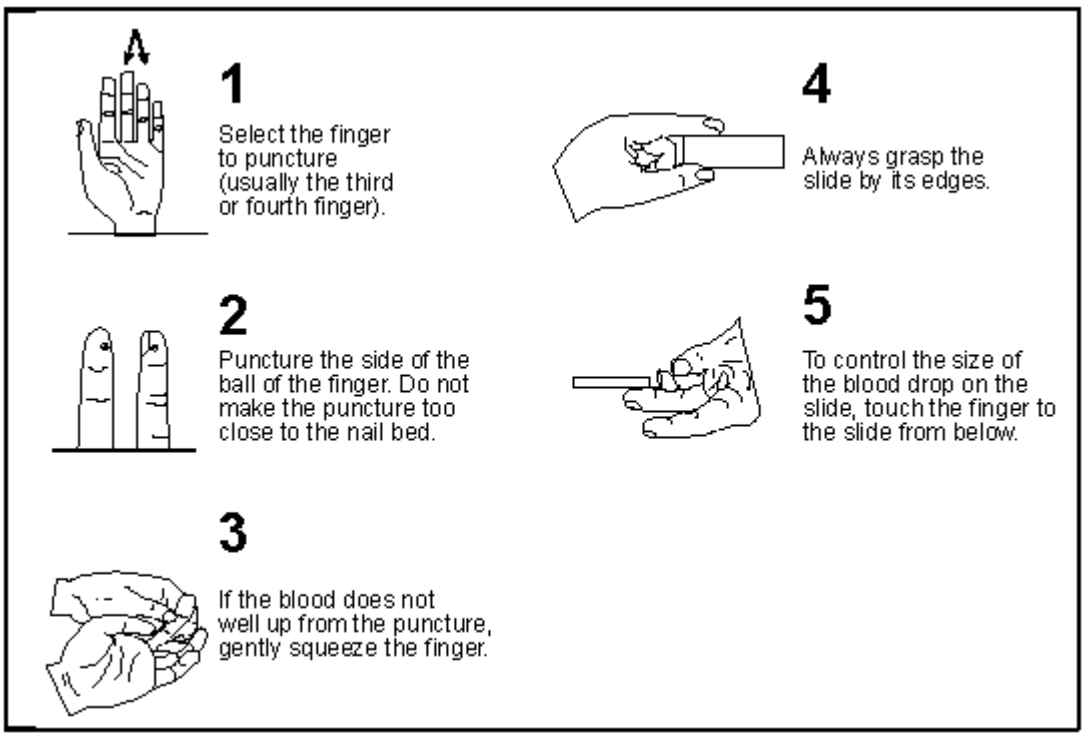
1. Alcohol solution (disinfectant)
2. Cotton balls
3. Sterile lancets
4. Glass slides
5. Methanol solution
6. Giemsa/Wright/or MayGrünwald stain

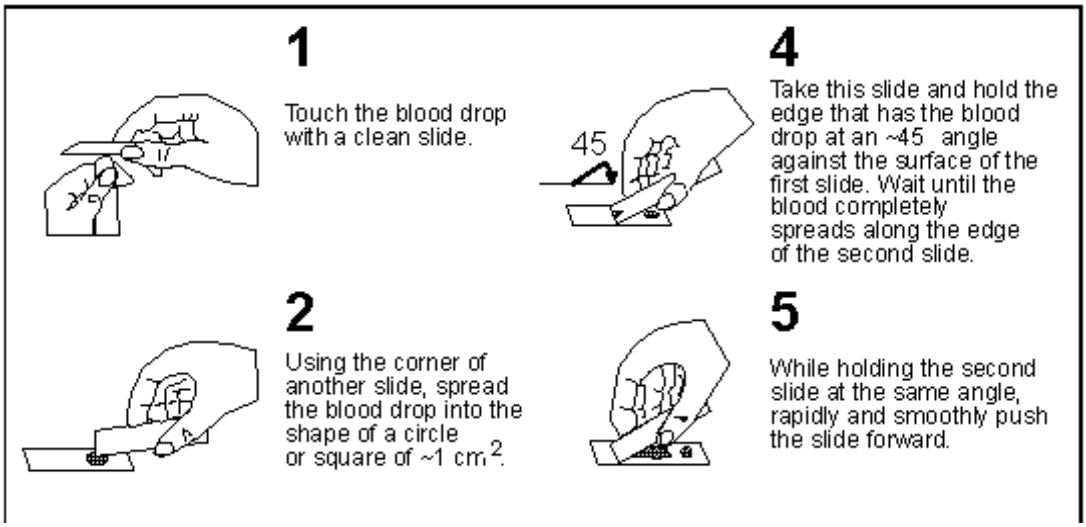
Method:

1. Prepare a blood smear on a glass slide as shown by the instructor (and figure 1)
    a. **Note: Make sure to disinfect the finger before puncturing**

2. To enhance your chances for a successful finger puncture the first time, warm your finger if it is cold, shake your finger several times to dry the alcohol and force more blood into it.
    a. **Caution**: Make sure the used lancets are discarded immediately to disinfectant container to prevent subsequent injury.

Figure 1:





1. Air dry the smear
2. Fix with methanol for 1min
3. Stain it for 15 min
4. Observe the structures on microscope under 100X.
5. Draw all the blood cells in your results section

11.1.Parasitology-II

• Parasitic evaluation of stool specimen:

1. Microscopic evaluation with Wet-mount technique, and Lugol solution
2. Microscopic evaluation with Giemsa and Metylene Blue staining techniques

Introduction

Diagnosis of parasite infections often depends on observing parasite stages including ova, larva, or adult forms. Sample types include stools (most common), tissue, and blood. Stool samples should be free of antibiotics, barium, or other substances that inhibit parasite growth. Fecal specimens for parasitic examination should be collected before initiation of antidiarrheal therapy or antiparasitic therapy. The highest yield on hospitalized patients occurs when diarrhea is present on admission or within 72 hours of admission. The onset of diarrhea more than 72 hours after admission is usually caused by *Clostridium difficile* toxin rather than parasites or the usual stool pathogens.

To ensure that good specimens are provided for examination, it is important to note the following points:

1. A clean dry container must be used for the collection of faecal samples. Urine and water will destroy trophozoites, if present, and the presence of dirt also causes identification problems.
2. Ideally the specimen should be brought to the lab as soon as it is passed, to avoid deterioration of protozoa and alterations of the morphology of protozoa and helminths.
3. The specimen container should be clearly labelled with the patients name, date, and time of passage of the specimen.
4. An amount of stool adequate for parasite examination should be collected and a repeat sample requested if too little is supplied.
5. Diarrheal specimens, or those containing blood and mucus, should be examined promptly on arrival in the laboratory. The specimens may contain motile amoebic or flagellate trophozoites and may round up and thus be missed if examination is delayed. Where amoebic dysentery is suggested, the laboratory should be informed that a "hot stool" is being supplied so that it can be examined within twenty minutes of being passed.
6. If specimens cannot be examined as soon as they arrive, they should be put in the refrigerator.
7. Specimens from patients with HIV should be left in 10% formalin for hour before proceeding with parasite examination.

We have to make sure the following rules for an ideal evaluation:

1. Three grams of sample is required for most parasite analysis.
2. Stool should be free of urine, because urea inhibits some parasites.
3. **Fresh stools** are needed for amebae and flagellate **trophic forms**.
4. **Liquid stools** are best to detect tropic forms, while **formed stools** are best to detect ova and cyst forms.
5. Stools should not be frozen or allowed to stand at room temperature.
6. **Polyvinyl alcohol (PVA)** and **Sodium Acetate formalin (SAF)** can be used to preserve stool samples.
7. Gross examination of stool may detect **adult forms.**

Visual observation of the faecal sample:

It is important to observe the <u>macroscopic appearance</u> of the stool as this can give a clue to the type of organisms present.

Therefore the consistency; formed, unformed or liquid; the colour and the presence or absence of an exudate are reported. The presence of adult worms can also be seen in a freshly passed stool eg adult stages of *Ascaris lumbricoides* and *Enterobius vermicularis*. Proglottids of *Taenia* species can also be seen.

Materials and Methods:

1. **Simple Method**:
    a. **Materials:**
        i. Stool sample in a container
        ii. Physiological saline solution (0.9% NaCl solution)
        iii. Lugol solution
        iv. Glass slides and cover slips, Stirring stick
        v. Disinfectant container
    b. **Evaluation of stool sample with Physiological saline;**
        i. Put one or two drops of physiological saline solution onto the glass slide.
        ii. With the aid of an stirring stick take a small sample of stool from one or more areas.
        iii. Mix them homogenously and cover it with cover slip.
        iv. Start scaning all the areas of the cover slip and observe suspected areas with 40X magnification. (With this method motile trophosoites are easilly detected.)
    c. Evaluation with Lugol solution:
        i. Repeat the same procedure with Lugol solution instead of physiological saline solution.
        ii. With this procedure parazite ova(egg) and cysts are much easily detected.
        iii. The glycogen vacuoles of the cysts are seen in brown, cytoplasm yellow and nuclei colorless.
        iv. Start scanning all the areas of the cover slip and observe suspected areas with 40X magnification
    d. Results:
        i. Draw your microscopic evaluations on your "**results section**" in your report.

2. **Method 2:**
    a. **Evaluation of a stool sample using permanent staining method:**
        i. **Materials:**
            1. **Methylene-Blue stain and Giemsa stain** will be used to evaluate morphologies of eggs and trophozoite form.
            2. **Method:**
                a. Make 2 thin film of faeces.
                b. Allow to air dry
                c. Fix in methanol for 1 minute
                d. Tip off the methanol
                e. Flood one of the slides with Giemsa stain and the other one with Methylene Blue stain
                f. Stain each for 20-25 minutes
                g. Run tap water on to the slide to float off the stain and to prevent deposition of precipitate on to the film
                h. Allow to drain, and dry
                i. Examine the film using the oil immersion objective.
                    i. Parasite nuclei and structures containing chromatin stain red**.**
                    ii. Cytoplasm stains **bluish-grey.**
                    iii. Leucocyte nuclei stain **purple.**
                    iv. Yeasts and bacteria stain **dark blue.**
                j. Draw your findings in your "**results section**" of your lab report.

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