Al-Anbar University

College of Sciences

Biology department

Subject name: Microbial Identification

Educational level: Master

Lecture title: Cultivation of Bacteria and fungi

Subject teacher

Assistant prof. Dr. Jamal A. Rahman

Chapter 4

Cultivation of Bacteria and Fungi

Introduction

The cultivation, or growth, of bacteria and fungi is necessary for subsequent isolation, identification, and determination of antibiotic susceptibility. You must understand an organism's specific nutritional and environmental requirements if your efforts at cultivation are to be successful. You must also use good aseptic techniques to prevent contamination. This chapter discusses the cultivation of various bacteria and fungi on a variety of culture media.

SAMPLE COLLECTION

Environmental Samples

Microorganisms, such as bacteria and fungi, are present in air, soil, water, and the laboratory environment. To demonstrate the presence of microbes in air, leave an agar plate open for a period of time. Growth will appear on the agar surface. To demonstrate their presence in soil and water, place a small amount of sample directly on an agar plate and then use a **sterile swab** (Figure 4.1a) to spread the sample over the agar surface. Similarly, the presence of bacteria and fungi in the laboratory can be demonstrated by rubbing a surface with a wet, sterile swab and then rubbing the swab back and forth across the surface of an agar plate.

Clinical Samples

Bacteria from human body surfaces are also collected using sterile swabs (Figure 4.1b–d). Swabs can collect bacteria from external (skin) and internal (throat, reproductive tract) body surfaces. After sampling, the swab is either used to inoculate an appropriate medium or placed into a holding medium until inoculation.

Blood from the human body is inoculated into **culture bottles** (Figure 4.2) to test for the presence

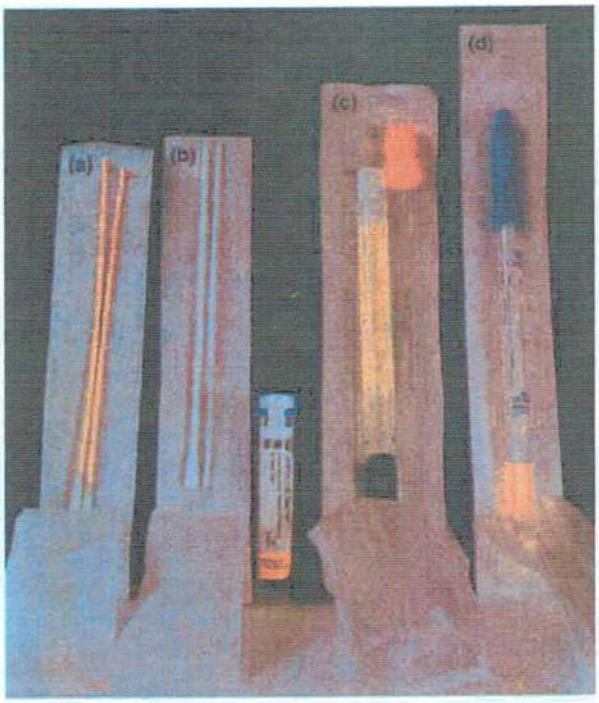


FIGURE 4.1 Sample collection swabs: (a) sterile cotton-tipped applicators; (b) STD swab collection and transport kit; (c) STAR collection and transport system; (d) Culturette® swab collection and transport system.

of bacteria. After incubation, culture bottles are examined for signs of bacterial growth, such as turbidity and gas production. Growth from bottles can then be inoculated into an appropriate medium for further study.

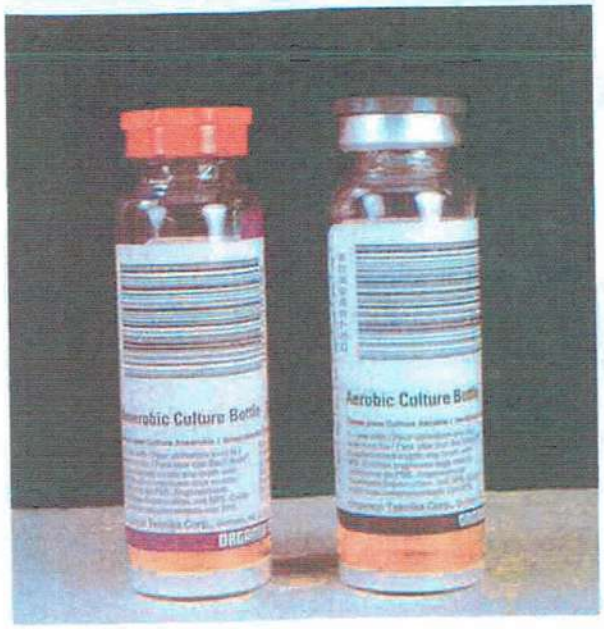


FIGURE 4.2 Anaerobic (left) and aerobic (right) blood-culture bottles.

FIGURE 4.3 A sputum collection kit. Sputum is collected in the sterile graduated tube. After collection, the tube is capped.

Similarly, sputum from the lungs, mid-stream urine, and stool are collected in various types of sterile containers (Figures 4.3–4.5). After collection, each of these samples is then inoculated into an appropriate medium.

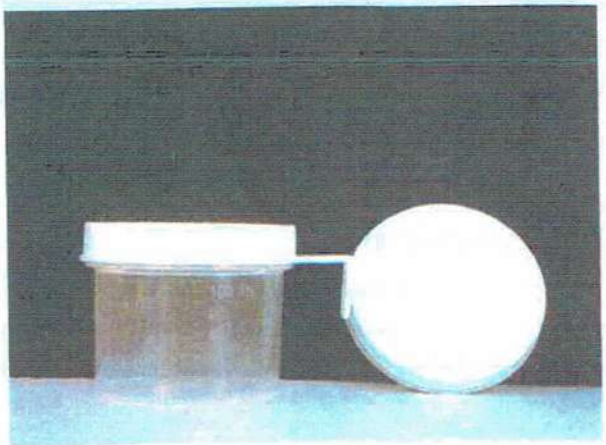


FIGURE 4.4 A mid-stream urine collection kit. After collection, the sterile cup is capped.

FIGURE 4.5 A stool collection kit. The scoop is used to transfer stool into the sterile container, which is then capped.

INCUBATION OF INOCULATED MEDIA

Temperature Considerations

Rapid growth of bacterial samples is desirable, especially for clinical isolates, because patient treatment often must await the results of bacterial identification and antibiotic susceptibility tests. Rapid growth is assured by incubating media at a temperature that is optimal for bacterial growth, which can be different for different bacteria.

Although there are bacteria from natural environments that are **psychrophilic** (requiring cold temperatures) or **thermophilic** (requiring hot temperatures), those from the human body are **mesophilic,** meaning that they require a temperature range of 20–40°C for growth. Bacteria from the human body have an optimal temperature for growth of around 35°C. Because this temperature is 10–15°C above room temperature, media must be placed in an incubator if optimal growth is to occur (Figure 4.6). Notice in Figure 4.7 that an isolate of *Staphylococcus aureus* has clearly visible growth after 24 hours at 35°C. However, growth is barely visible after 24 hours at 25°C, and no growth is visible after 24 hours at 5°C.

In the example in Figure 4.7, why is growth most visible at 35°C? If bacteria are placed at two temperatures within their growth range, they will

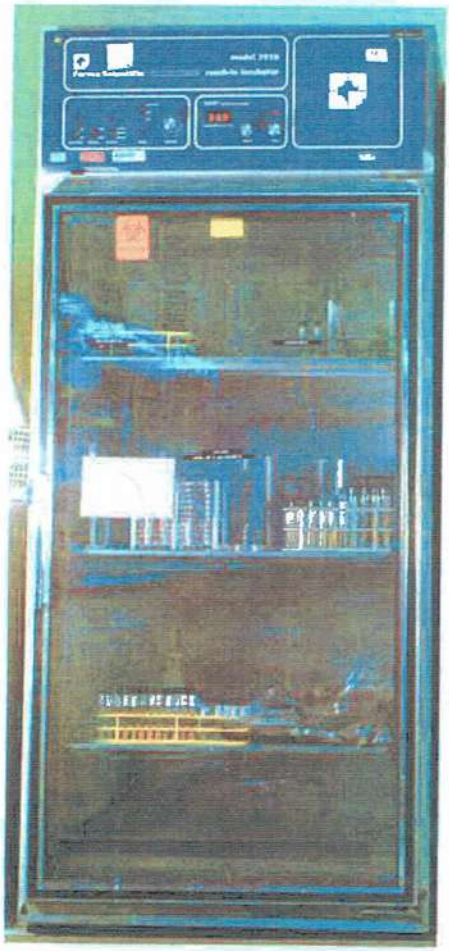


FIGURE 4.6 A large incubator containing bacterial cultures.

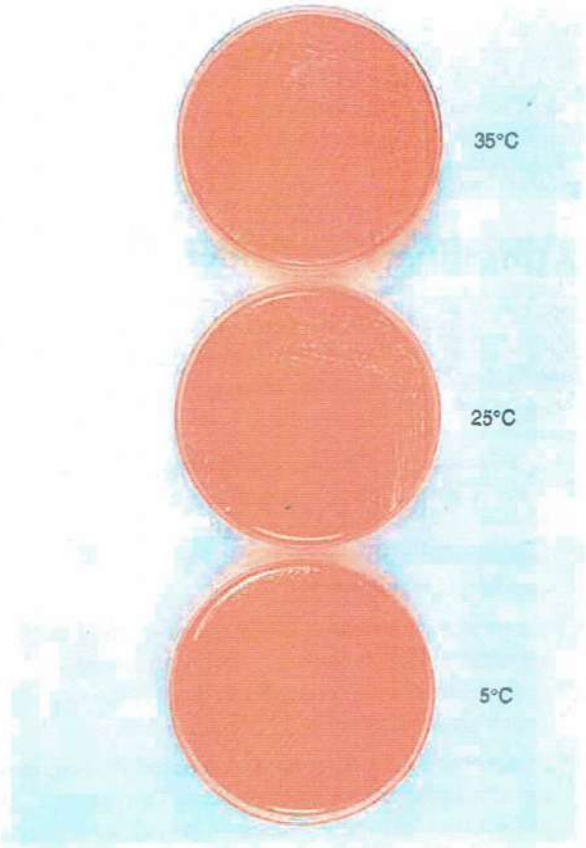
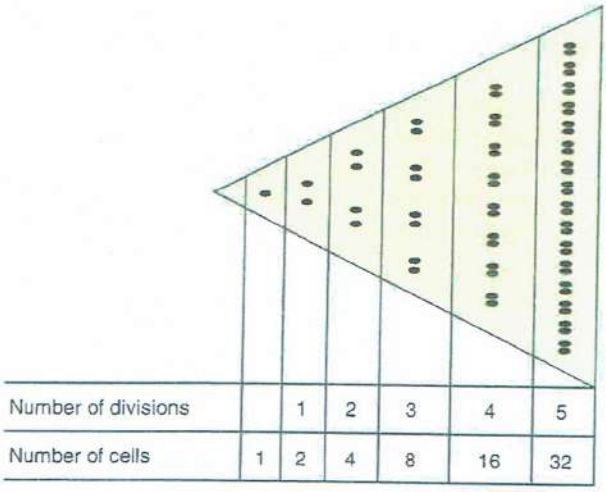


FIGURE 4.7 *Staphylococcus aureus* growth on blood agar after 24 hours at 35°, 25°, and 5°C. Growth is most visible at the optimal temperature of 35°C.



Number of divisions		1	2	3	4	5
Number of cells	1	2	4	8	16	32

FIGURE 4.8 The growth of bacterial cells: the relation of number of cells to number of divisions.

typically divide more often at the higher temperature. As illustrated in Figure 4.8, a greater number of divisions results in a greater number of cells, so colonies will appear more quickly on an agar plate maintained at higher temperatures within the growth range.

Atmospheric Considerations

Bacteria that are **aerobes** and **facultative anaerobes** grow well in air, which contains oxygen. However, some bacteria have special atmospheric requirements for growth, such as reduced oxygen or a lack of oxygen.

- *Neisseria gonorrhoeae* and *Haemophilus influenzae* grow best in an atmosphere that is low in oxygen and enriched in carbon dioxide. This **capnophilic** atmosphere can be achieved with a **candle jar** (Figure 4.9).
- *Campylobacter jejuni*, an intestinal pathogen, requires a **microaerophilic** environment for growth, one with reduced oxygen. This can be provided by a Bio-Bag™ (Figure 4.10).
- **Anaerobic** bacteria, such as *Clostridium*, require an atmosphere without oxygen. This type of atmosphere can be provided by either an **anaerobic jar** (Figure 4.11) or an **anaerobic hood** (Figure 4.12).

FIGURE 4.9 A candle jar. The candle is lighted just before sealing the jar. As the candle burns, it consumes oxygen and produces carbon dioxide. When most of the oxygen in the jar is used up, the flame goes out.

FIGURE 4.10 A Bio-Bag™. The gas generator tube (right), when broken, combines a chemical tablet with hydrochloric acid to produce a microaerophilic atmosphere.

FIGURE 4.11 An anaerobic jar. The contents of the GasPak *Plus*™ envelope, when activated by water, consume oxygen and produce an atmosphere of hydrogen and carbon dioxide.

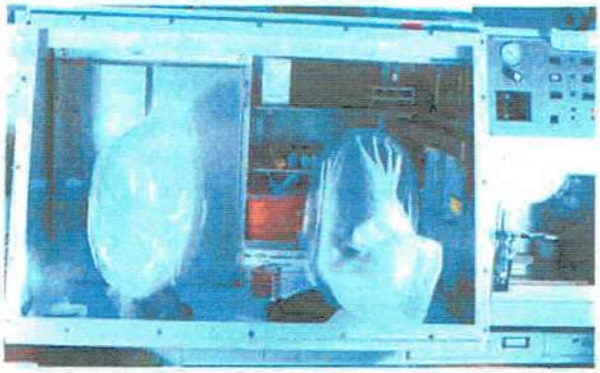


FIGURE 4.12 An anaerobic hood for the transfer and incubation of anaerobic bacteria.

EXAMINATION OF GROWTH ON AGAR MEDIA

A variety of agar media are used for the cultivation of bacteria and fungi. The results of environmental and clinical samples inoculated onto various media are described next.

Growth of Organisms from the Environment

A variety of bacteria and fungi are present in the environment. In some situations, microbiologists seek to culture these organisms. In other cases these organisms are considered environmental contaminants that must be kept out of culture media through the use of good **aseptic techniques.** Nutrient agar and Sabouraud dextrose agar can be used to demonstrate the presence of these organisms in the environment.

Nutrient agar is used to demonstrate bacteria in the environment. This medium, which contains beef extract and peptone, supports a wide variety of heterotrophic bacteria, such as those present in air (Figure 4.13), in soil, and on the laboratory floor (Figure 4.14).

Fungi are inhibited by large populations of bacteria on nutrient agar and will only grow if few

FIGURE 4.13 Bacteria from air inside the laboratory (left) and outside the laboratory (right) on nutrient agar. Each plate was left open for 30 minutes. The greater number of bacteria on the plate left open outside the lab is due to the greater number of particles in the outside air.

bacteria are present in a sample (Figure 4.15). For this reason, nutrient agar is not reliable for routine cultivation of fungi.

Sabouraud dextrose agar (SDA) contains a high concentration of dextrose and has an acidic pH of 5.6. This medium is often supplemented with antibiotics to inhibit bacterial growth, making this medium selective for fungi (Figure 4.16). Sabouraud dextrose agar can be used to demonstrate the presence of fungi in air (Figure 4.17) and on the laboratory floor (Figure 4.18).

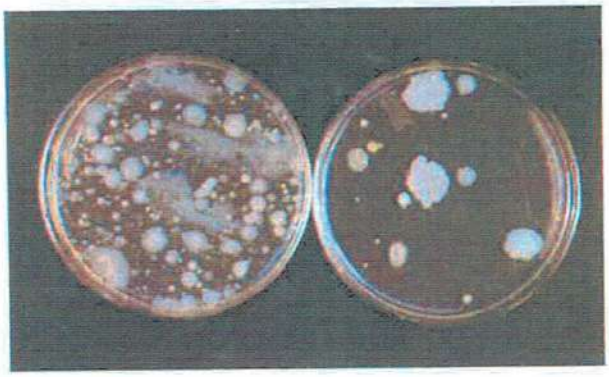


FIGURE 4.14 Bacteria from the soil (left) and from the laboratory floor (right) on nutrient agar. Notice the high density of bacteria in soil, some of which are transferred to the laboratory floor by shoes.

FIGURE 4.16 Nutrient agar (left) and Sabouraud dextrose agar (right) inoculated with the same soil dilution. Notice that Sabouraud dextrose agar inhibited the growth of most bacteria while supporting the growth of fungi.

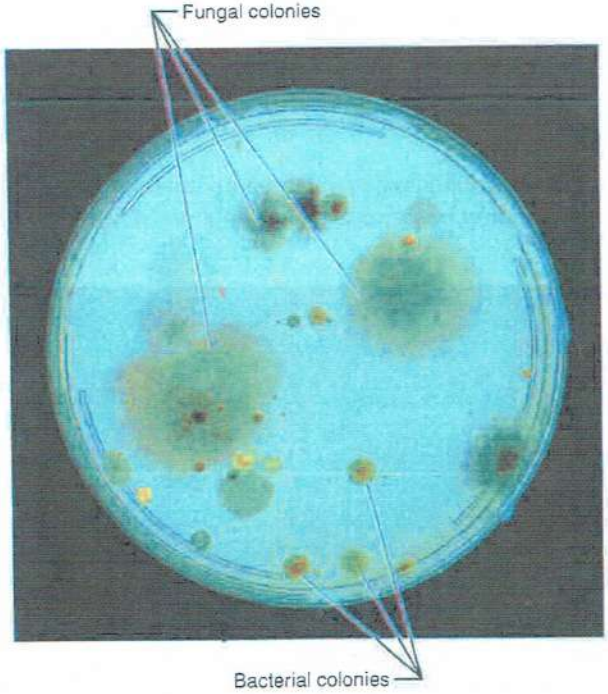


FIGURE 4.15 Bacteria and fungi from the laboratory floor on nutrient agar. Fungi grow on nutrient agar only when few bacteria are present.

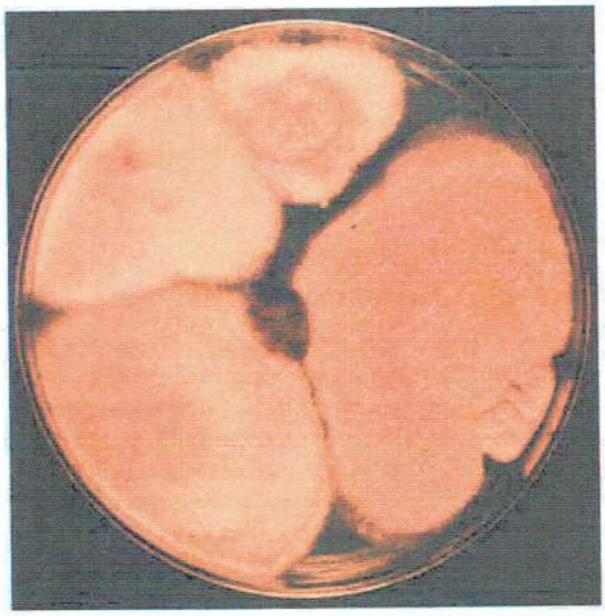


FIGURE 4.17 Fungi from laboratory air on Sabouraud dextrose agar. The plate was left open for 30 minutes.

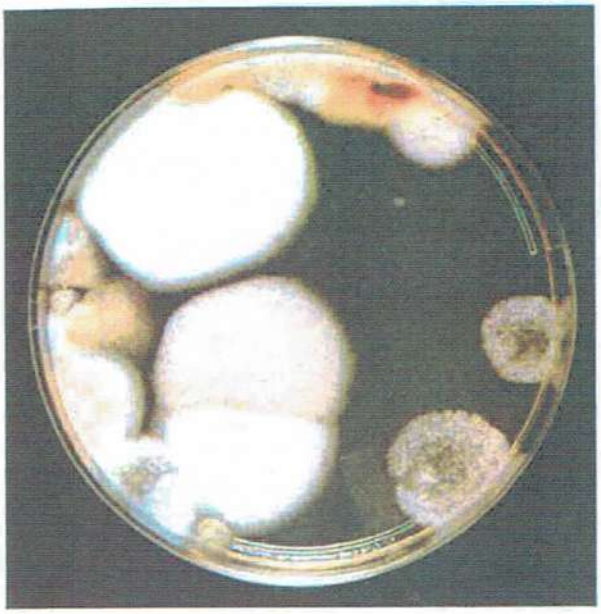


FIGURE 4.18 Fungi from laboratory floor on Sabouraud dextrose agar.

Growth of Organisms from Clinical Samples

ENRICHED AGARS Enriched media such as blood agar and chocolate agar, which are supplemented with blood, are routinely used to culture pathogenic bacteria from clinical samples.

Blood agar contains a basal medium, such as brain/heart infusion or tryptic soy, and 5–10% sheep, horse, or rabbit blood. This enriched agar is often used as a primary isolation medium for obtaining clinical isolates from sputum (Figure 4.19), the throat (Figure 4.20), and skin (Figure 4.21). Blood agar is also useful as a differential agar, because it differentiates bacteria on the basis of blood cell hemolysis. **Hemolysis** is useful in the identification of a number of clinical isolates. The three types of hemolysis are as follows:

- Some bacteria completely lyse, or break down, red blood cells, resulting in a clear area around colonies. This type of hemolysis, called **beta-hemolysis**, is displayed by *Streptococcus pyogenes* (Figure 4.22).
- Other bacteria only partially lyse red blood cells, resulting in a greenish discoloration around colonies. This type of hemolysis, called **alpha-hemolysis**, is demonstrated by *Streptococcus pneumoniae* (Figure 4.23).
- Other bacteria do not lyse red blood cells, so the appearance of the agar remains the same. This type of result, called **gamma-hemolysis**, is typical of *Enterococcus faecalis* (Figure 4.24).

Chocolate agar is used as a primary isolation medium (Figure 4.25) and to culture certain bacteria,

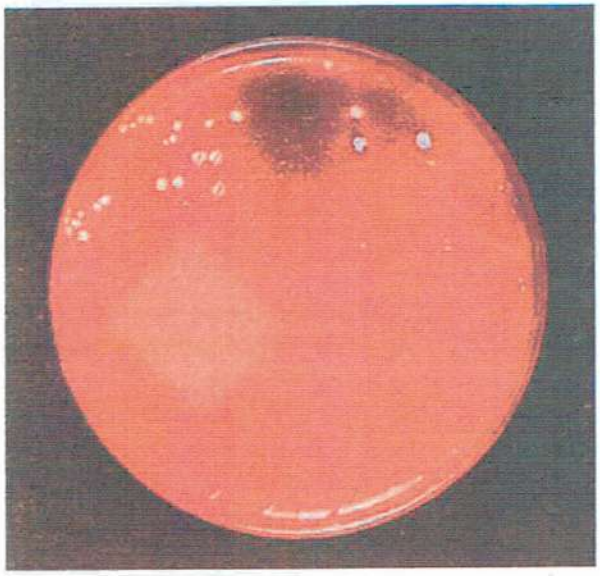


FIGURE 4.19 Bacterial growth on blood agar inoculated with sputum.

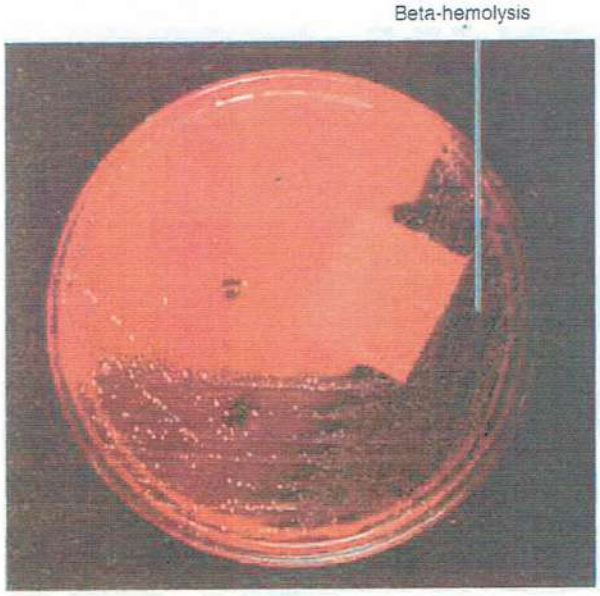


FIGURE 4.20 Bacterial growth on blood agar inoculated with a throat swab. Notice the clearing around growth, a reaction on blood agar called beta-hemolysis.

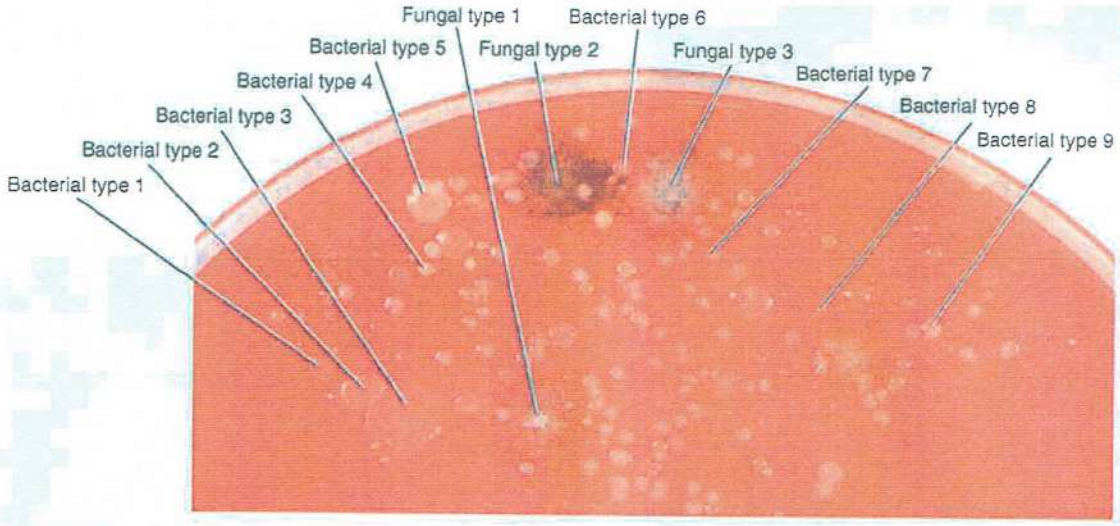


FIGURE 4.21 Bacterial and fungal growth on blood agar inoculated with a skin swab. Notice the great variety of bacteria and fungi that live on the skin.

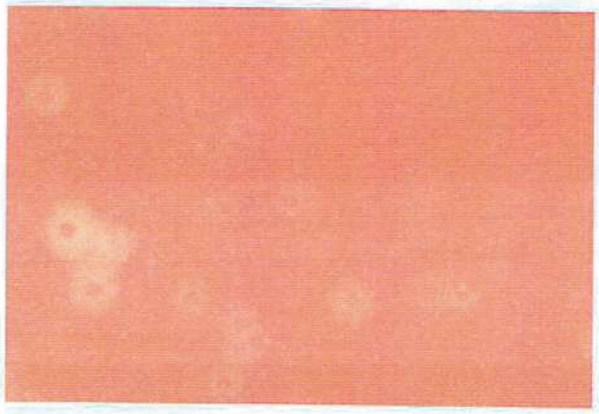


FIGURE 4.22 Beta-hemolysis, indicated here by a clearing around *Streptococcus pyogenes* colonies on blood agar (6×).

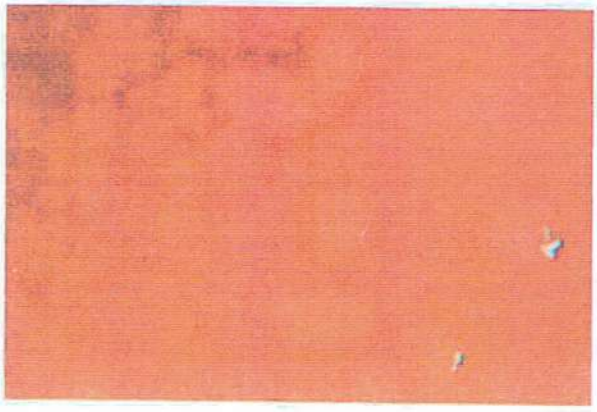


FIGURE 4.23 Alpha-hemolysis, indicated here by a green color around *Streptococcus pneumoniae* colonies on blood agar (6×).

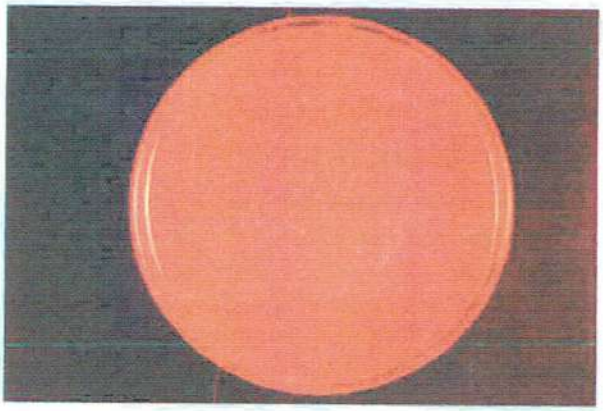


FIGURE 4.24 Gamma-hemolysis, indicated here by a lack of clearing or other change around colonies of *Enterococcus faecalis* on blood agar.

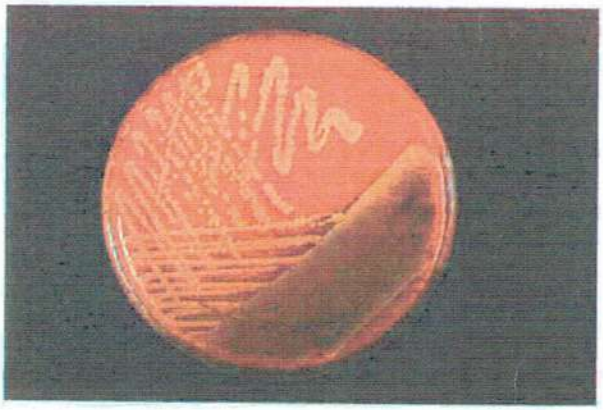


FIGURE 4.25 Bacterial growth on chocolate agar inoculated with liquid from a blood culture bottle. Notice the "chocolate brown" color of the medium.

such as *Neisseria gonorrhoeae* and *Haemophilus influenzae*. It contains a peptone base and blood. Heating the medium lyses red blood cells, which releases nutrients and results in a "chocolate brown" color.

SELECTIVE/DIFFERENTIAL AGARS FOR GRAM-POSITIVE BACTERIA

Bile esculin agar contains beef extract, peptone, esculin, bile, and ferric citrate and is differential for group D streptococci, including *Enterococcus faecalis*. These bacteria utilize esculin in the presence of bile, producing a product that reacts with the ferric citrate in the medium to form a dark-brown color (Figure 4.26).

Colistin-nalidixic acid (CNA) agar contains colistin and nalidixic acid, both inhibitory to Gram-negative bacteria. Blood is also added to the medium. This agar selects for Gram-positive bacteria, such as *Staphylococcus aureus*, while inhibiting Gram-negative bacteria, such as *Escherichia coli* (Figure 4.27).

Mannitol salt agar (MSA) contains 7.5% NaCl, which is inhibitory to most bacteria. However, staphylococci grow on this agar and can be differentiated on the basis of mannitol fermentation. Pathogenic staphylococci, such as *Staphylococcus aureus*, ferment mannitol to form acidic products that lower the pH of the medium. When this happens, the phenol red pH indicator in the medium turns from red to yellow (Figure 4.28). Nonpathogenic staphylococci, such as *Staphylococcus epidermidis*, do not ferment mannitol, so no color change occurs.

Phenylethyl alcohol agar (PEA) contains phenylethyl alcohol, which is inhibitory to Gram-negative bacteria. Therefore, this agar selects for

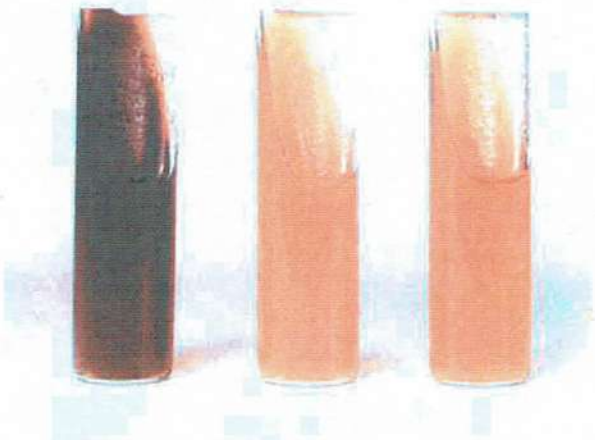


FIGURE 4.26 Bile esculin agar tubes inoculated with *Enterococcus faecalis* (left), inoculated with *Proteus vulgaris* (center), and left uninoculated (right). The dark brown color at left is characteristic of group D streptococci on this medium.

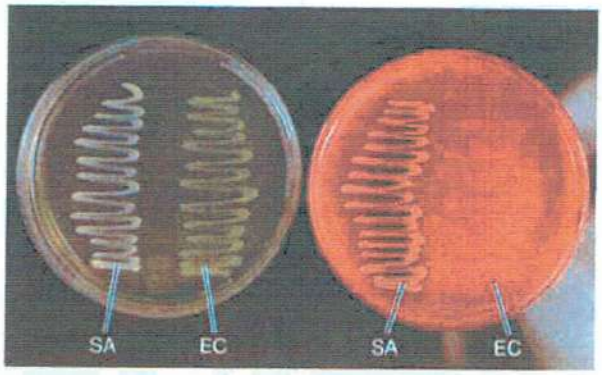


FIGURE 4.27 Nutrient agar (left) and colistin-nalidixic acid agar (right), each inoculated with *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Gram-negative *E. coli* is inhibited on CNA agar, while Gram-positive *S. aureus* is not. This demonstrates the Gram-positive selective nature of CNA agar.

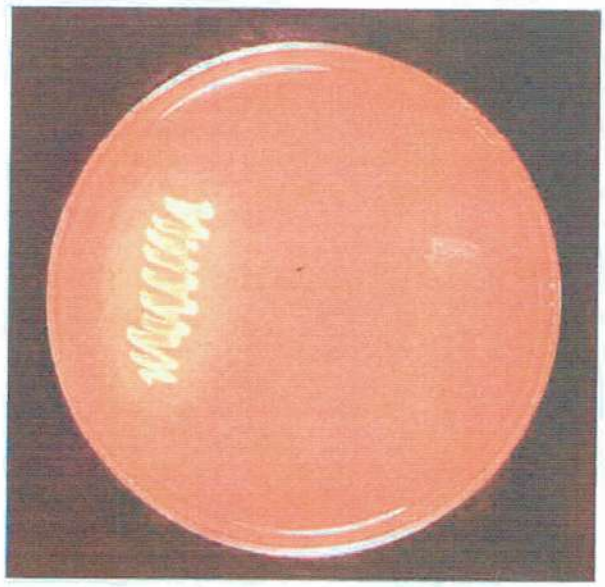


FIGURE 4.28 Mannitol salt agar inoculated with *Staphylococcus aureus* (at left) and *Staphylococcus epidermidis* (at right). Both species of staphylococci grow on this medium, but only *S. aureus* ferments mannitol to form acidic products that change the color of the agar to yellow.

Gram-positive bacteria, such as *Staphylococcus aureus,* while inhibiting Gram-negative bacteria, such as *Escherichia coli* (Figure 4.29).

SELECTIVE/DIFFERENTIAL AGARS FOR GRAM-NEGATIVE BACTERIA

Acetamide agar contains acetamide and is used as a differential medium for *Pseudomonas aeruginosa,* a frequent cause of urinary tract infections. This organism utilizes acetamide, producing alkaline end products that raise the pH of the medium. When this happens, the phenol red pH indicator in the medium turns red (Figure 4.30).

Endo agar contains the dye basic fuchsin, which is inhibitory to Gram-positive bacteria. Therefore, this medium selects for Gram-negative bacteria, such as *Escherichia coli,* while inhibiting Gram-positive bacteria, such as *Staphylococcus aureus* (Figure 4.31). Endo agar also differentiates lactose-fermenting bacteria from non-lactose-fermenting bacteria. Lactose-fermenting bacteria, such as *Escherichia coli,* appear red, while non-lactose-fermenting bacteria, such as *Shigella flexneri,* appear colorless (Figure 4.32). Notice in Figure 4.33 that

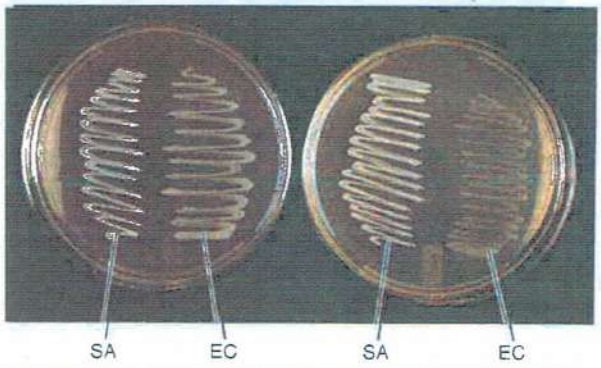


FIGURE 4.29 Nutrient agar (left) and phenylethyl alcohol agar (right), each inoculated with *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Gram-negative *E. coli* exhibits reduced growth on PEA, while Gram-positive *S. aureus* exhibits good growth. This demonstrates the Gram-positive selective nature of PEA agar.

FIGURE 4.30 Acetamide agar tubes inoculated with *Pseudomonas aeruginosa* (left), inoculated with *Proteus vulgaris* (center), and left uninoculated (right). The red color at left differentiates *P. aeruginosa* from other bacteria on this agar.

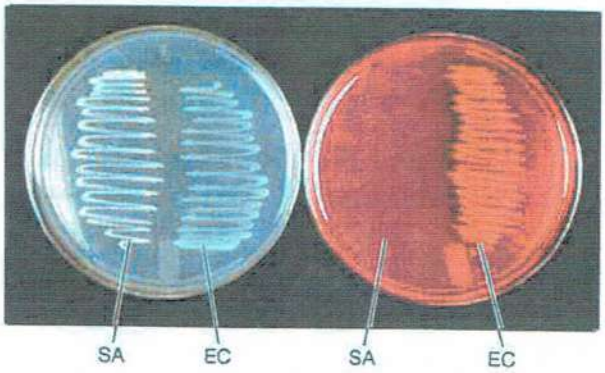


FIGURE 4.31 Nutrient agar (left) and endo agar (right), each inoculated with *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Gram-positive *S. aureus* is inhibited on endo agar, while Gram-negative *E. coli* is not. This demonstrates the Gram-negative selective nature of endo agar.

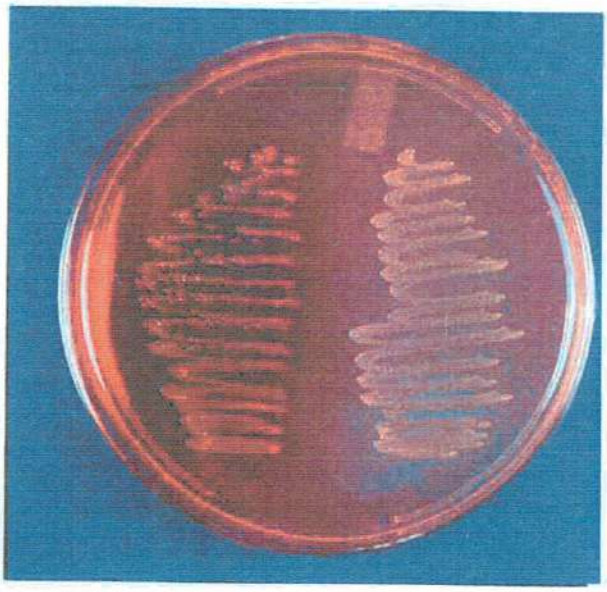


FIGURE 4.32 Endo agar inoculated with lactose-fermenting *Escherichia coli* (at left) and non-lactose-fermenting *Shigella flexneri* (at right). Lactose-fermenting bacteria appear red, while non-lactose-fermenting bacteria appear colorless. This demonstrates the differential nature of endo agar.

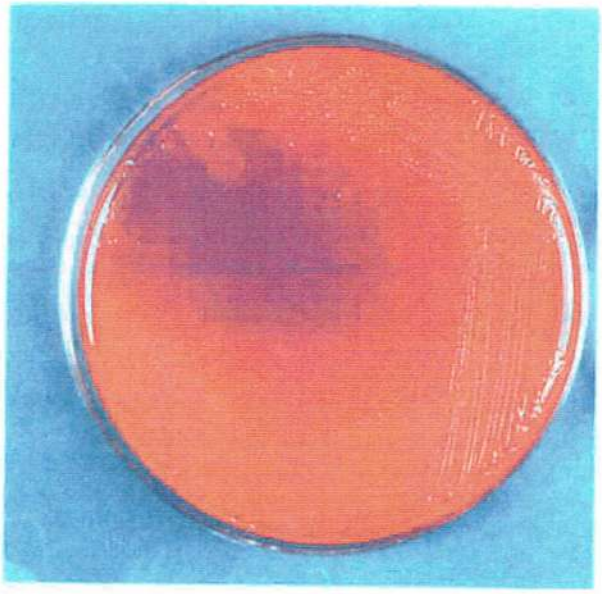


FIGURE 4.33 Endo agar streaked with lactose-fermenting *Escherichia coli*. The red color and metallic sheen are characterisic of *E. coli* on this agar.

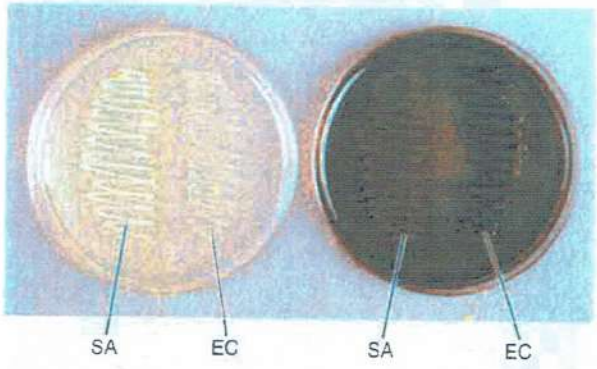


FIGURE 4.34 Nutrient agar (left) and eosin-methylene blue agar (right), each inoculated with *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Gram-positive *S. aureus* exhibits reduced growth on EMB agar, while Gram-negative *E. coli* exhibits good growth. This demonstrates the Gram-negative selective nature of EMB agar.

Escherichia coli, a lactose fermenter, produces a characteristic red color with a metallic sheen on endo agar.

Eosin-methylene blue (EMB) agar contains bile salts and the dyes eosin and methylene blue, all inhibitory to Gram-positive bacteria. Therefore, this medium selects for Gram-negative bacteria, such as *Escherichia coli*, while inhibiting Gram-positive bacteria, such as *Staphylococcus aureus* (Figure 4.34). EMB agar also differentiates lactose-fermenting bacteria from non-lactose-fermenting bacteria. Lactose-fermenting bacteria, such as *Escherichia coli*, often appear dark with a green metallic sheen, while non-lactose-fermenting bacteria, such as *Shigella flexneri*, appear colorless (Figure 4.35). The characteristic dark color and green metallic sheen of *E. coli* on this medium is apparent in Figure 4.36. *Enterobacter aerogenes*, also a lactose fermenter, produces characteristic dark-centered colonies on EMB agar (Figure 4.37).

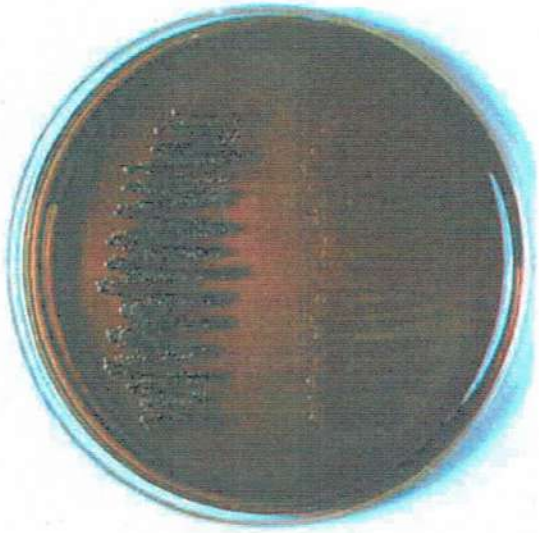


FIGURE 4.35 Eosin-methylene blue agar inoculated with lactose-fermenting *Escherichia coli* (at left) and non-lactose-fermenting *Shigella flexneri* (at right). Lactose-fermenting bacteria appear dark, or dark with a green metallic sheen, while non-lactose-fermenting bacteria appear colorless. This demonstrates the differential nature of EMB agar.

MacConkey (MAC) agar contains bile salts and crystal violet, both inhibitory to Gram-positive bacteria. Therefore, this medium selects for Gram-negative bacteria, such as *Escherichia coli*, while inhibiting Gram-positive bacteria, such as *Staphylococcus aureus* (Figure 4.38). This medium also differentiates lactose-fermenting bacteria from non-lactose-fermenting bacteria. Lactose-fermenting bacteria, such as *Escherichia coli*, appear pink or red, while non-lactose-fermenting bacteria, such as *Shigella flexneri*, appear colorless (Figure 4.39). Notice that the lactose fermenter *Enterobacter aerogenes* appears red on MAC agar (Figure 4.40).

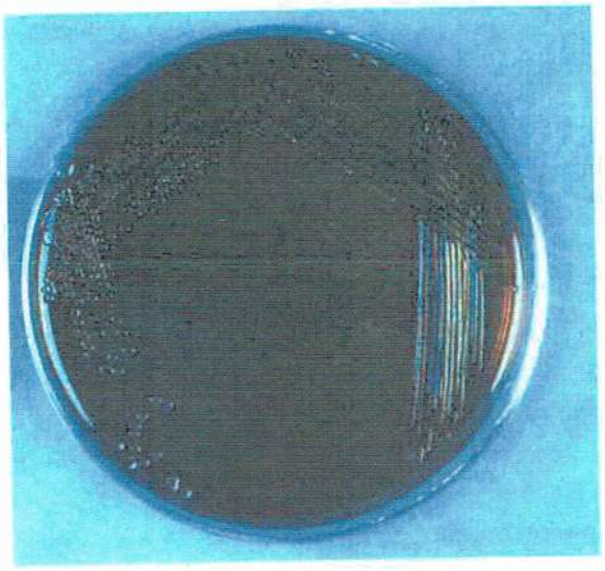


FIGURE 4.36 Eosin-methylene blue agar streaked with lactose-fermenting *Escherichia coli*. The dark color and green metallic sheen are characteristic of *E. coli* on this agar.

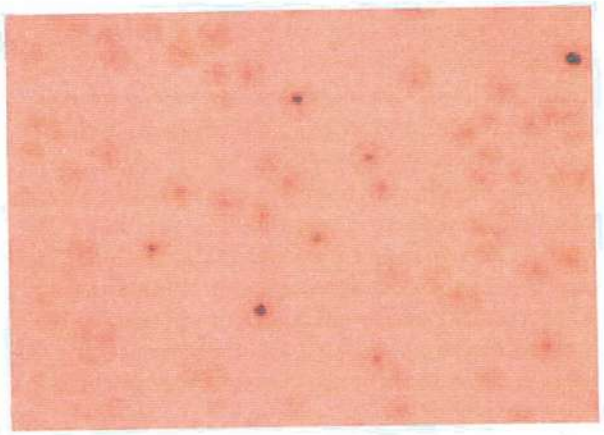


FIGURE 4.37 Eosin-methylene blue agar inoculated with lactose-fermenting *Enterobacter aerogenes*. Colonies with dark centers are characteristic of *E. aerogenes* on this agar (3×).

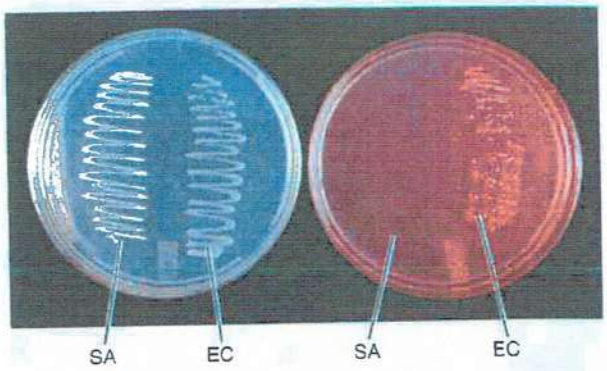


FIGURE 4.38 Nutrient agar (left) and MacConkey agar (right), each inoculated with *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Gram-positive *S. aureus* is inhibited on MAC agar, while Gram-negative *E. coli* is not. This demonstrates the Gram-negative selective nature of MAC agar.

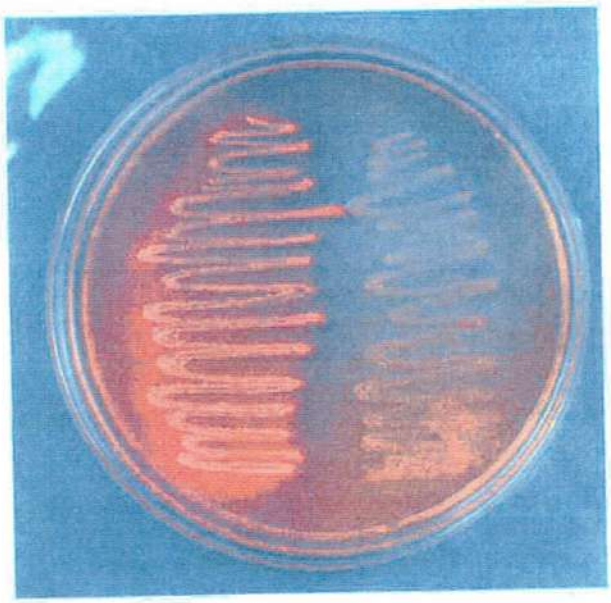


FIGURE 4.39 MacConkey agar inoculated with lactose-fermenting *Escherichia coli* (at left) and non-lactose-fermenting *Shigella flexneri* (at right). Lactose-fermenting bacteria appear pink or red, while non-lactose-fermenting bacteria appear colorless. This demonstrates the differential nature of MAC agar.

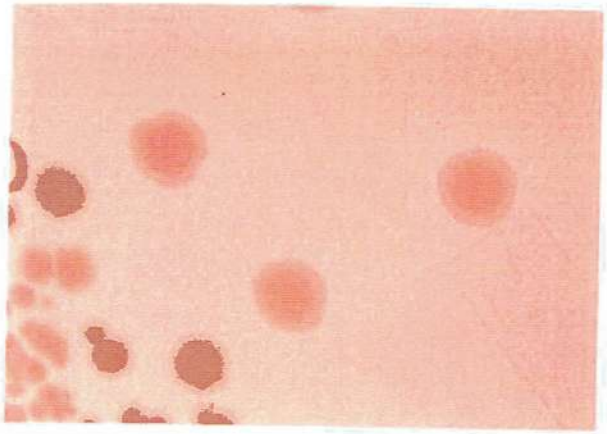


FIGURE 4.40 MacConkey agar inoculated with lactose-fermenting *Enterobacter aerogenes*. Red colonies are characteristic of *E. aerogenes* on this agar (6×).

BIPLATES: COMBINING GRAM-POSITIVE AND GRAM-NEGATIVE SELECTIVE AGARS A biplate contains a central divider that allows two media to be placed in a single petri dish. A biplate often combines Gram-positive and Gram-negative selective agars so that both bacterial types can be cultured on a single plate. A CNA/MAC biplate is often used in clinical laboratories for urine samples (Figure 4.41), because both Gram-positive and Gram-negative bacteria can cause urinary tract infections.

SELECTIVE/DIFFERENTIAL AGARS FOR PATHOGENIC ENTERICS **Hektoen enteric (HE) agar** contains a high concentration of bile salts and the dyes brom thymol blue and acid fuchsin, which are all inhibitory to Gram-positive bacteria and some Gram-negative enterics. Therefore, this medium is useful for culturing species of *Salmonella* and *Shigella*, bacteria that cause a variety of gastrointestinal illnesses in humans. This agar also differentiates nonpathogenic enterics from pathogenic enterics on the basis of lactose fermentation. Nonpathogenic enterics, such as *Escherichia coli*, ferment lactose and appear orange, while pathogenic enterics, such as *Shigella flexneri*, do not ferment lactose and appear green (Figure 4.42).

Salmonella-Shigella (SS) agar contains bile salts, sodium citrate, and brilliant green, which are all inhibitory to Gram-positive bacteria. This trait increases the yield of Gram-negative bacteria,

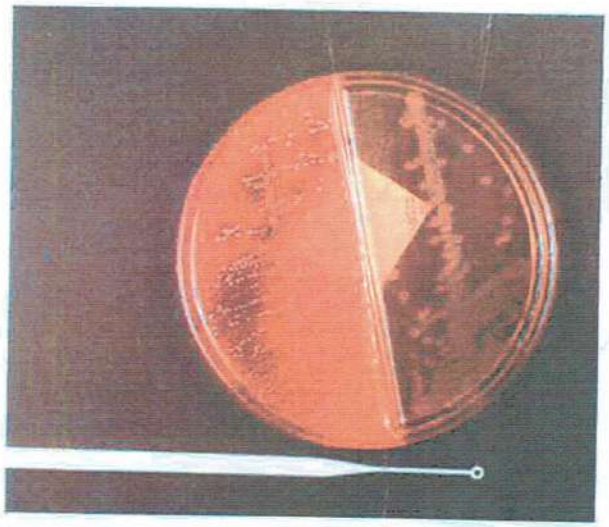


FIGURE 4.41 A biplate of colistin-nalidixic acid agar (at left) and MacConkey agar (at right) inoculated with urine. A 0.001-ml aliquot of urine was streaked onto each medium. Notice the growth on both sides of the plate, suggesting the presence of both Gram-positive and Gram-negative bacteria in this sample.

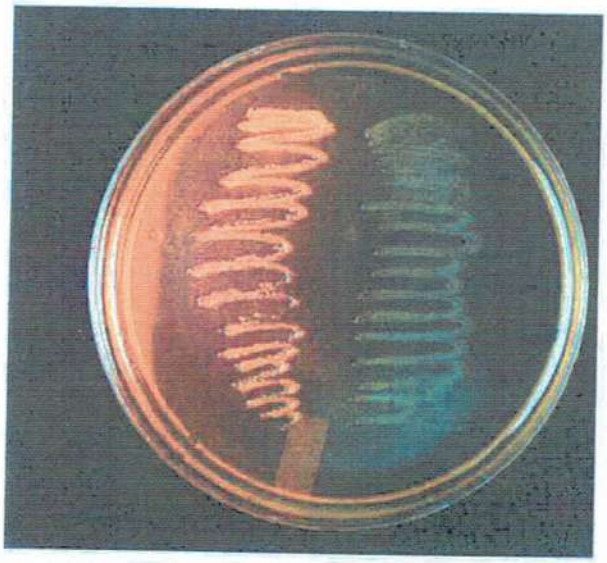


FIGURE 4.42 Hektoen enteric agar inoculated with the nonpathogenic enteric *Escherichia coli* (at left) and the pathogenic enteric *Shigella flexneri* (at right). Lactose-fermenting *E. coli* appear orange, while non-lactose-fermenting *S. flexneri* appear green. This demonstrates the differential nature of HE agar.

including *Salmonella* and *Shigella*. This agar also differentiates nonpathogenic enterics from pathogenic enterics on the basis of lactose fermentation. Nonpathogenic enterics, such as *Escherichia coli,* ferment lactose and appear red, while pathogenic enterics, such as *Salmonella typhimurium,* appear black (Figure 4.43). The black color of *S. typhimurium* colonies, which is apparent in Figure 4.44, is due to the production of hydrogen sulfide. Pathogenic enterics that do not produce hydrogen sulfide, such as *Shigella flexneri,* appear colorless on this medium.

Xylose-lysine-deoxycholate (XLD) agar contains deoxycholate, which is inhibitory to Gram-positive bacteria. This inhibition increases the growth of Gram-negative bacteria, including species of *Shigella*. This medium also differentiates nonpathogenic enterics from pathogenic enterics on the basis of lactose, sucrose, and xylose fermentation. The majority of nonpathogenic enterics, including *Escherichia coli,* ferment these compounds and appear yellow, while pathogenic enterics, such as *Shigella flexneri,* do not ferment them and therefore appear red (Figure 4.45).

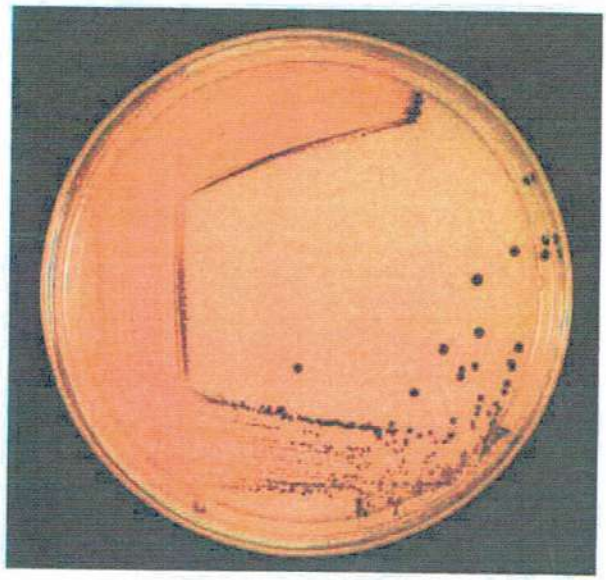


FIGURE 4.44 Salmonella-Shigella agar streaked with *Salmonella typhimurium*. The black color, characteristic of *S. typhimurium* on this agar, is due to the production of hydrogen sulfide, which reacts with iron in the medium to produce black iron sulfide.

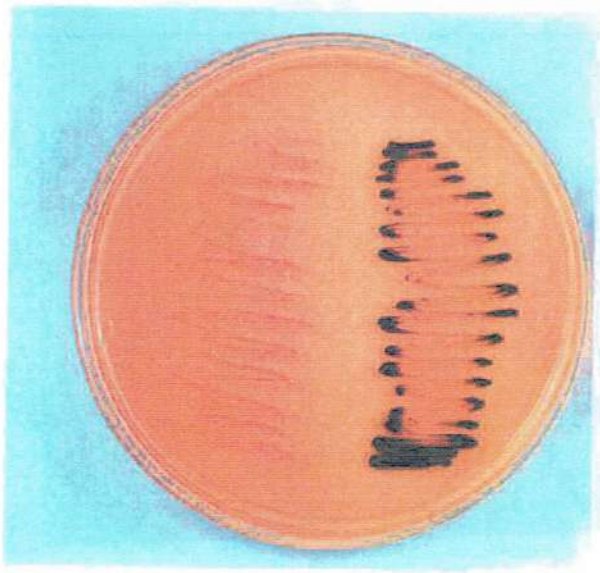


FIGURE 4.43 Salmonella-Shigella agar inoculated with the nonpathogenic enteric *Escherichia coli* (at left) and the pathogenic enteric *Salmonella typhimurium* (at right). Lactose-fermenting *E. coli* appear red, while non-lactose-fermenting *S. typhimurium* appear black. This demonstrates the differential nature of SS agar.

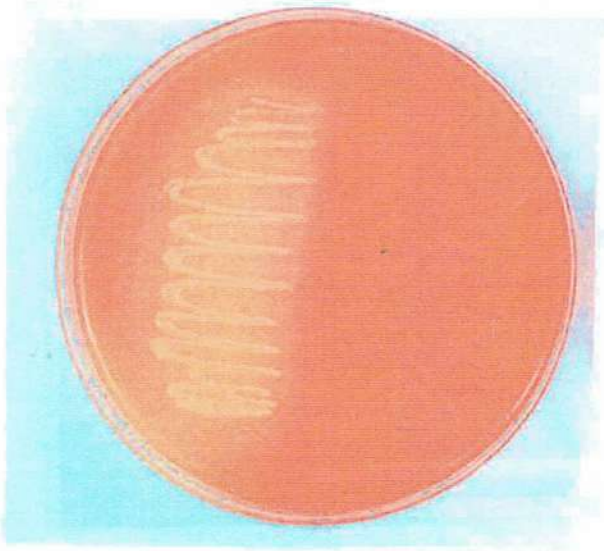


FIGURE 4.45 Xylose-lysine-deoxycholate agar inoculated with the nonpathogenic enteric *Escherichia coli* (at left) and the pathogenic enteric *Shigella flexneri* (at right). Lactose-fermenting *E. coli* appear yellow, while non-lactose-fermenting *S. flexneri* appear red. This demonstrates the differential nature of XLD agar.

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(2) FAIRBROTHER, R.W. (2013). **A Textbook of Bacteriology, Fourth Edition.**