Lab 12: Isolation and Identification of Enterobacteriaceae and Pseudomonas, Part 1

DISCUSSION

Labs 12 and 13 deal with opportunistic and pathogenic fermentative Gram-negative bacilli that are members of the bacterial family Enterobacteriaceae, as well as nonfermentative Gram-negative bacilli such as Pseudomonas and Acinetobacter.

A. ENTEROBACTERIACEAE: THE FERMENTATIVE, GRAM-NEGATIVE, ENTERIC BACILLI

Bacteria belonging to the family Enterobacteriaceae are the most commonly encountered organisms isolated from clinical specimens. The Enterobacteriaceae is a large diverse family of bacteria belonging to the order Enterobacteriales in the class Gammaproteobacter of the phylum Proteobacter. Medically important members of this family are commonly referred to as fermentative, Gram-negative, enteric bacilli, because they are Gram-negative rods that can ferment sugars. Many are normal flora of the intestinal tract of humans and animals while others infect the intestinal tract. Members of this family have the following characteristics in common:

1. They are Gram-negative rods (see Fig. 1)
2. If motile, they possess a peritrichous arrangement of flagella (see Fig. 2)
3. They are facultative anaerobes
4. With few exception, they are oxidase negative
5. All species ferment the sugar glucose but otherwise vary widely in their biochemical characteristics
6. Most reduce nitrates to nitrites.

For further information on the Gram-negative cell wall, see the following Learning Object in your Lecture Guide:

https://bio.libretexts.org/Learning_Objects/Laboratory_Experiments/Microbiology_Labs/Microbiology_Labs_II/Lab_12%3A_Isolate...
The Gram-negative cell wall, Unit 1, Section IIB2b

At least forty-four genera and over 130 species of Enterobacteriaceae have been recognized. Some of the more common clinically important genera of the family Enterobacteriaceae include:

- Salmonella
- Citrobacter
- Morganella
- Shigella
- Enterobacter
- Yersinia
- Proteus
- Serratia
- Edwardsiella
- Escherichia
- Klebsiella
- Providencia

Several genera of Enterobacteriaceae are associated with gastroenteritis and food-borne disease. These include:

- Salmonella,
- Shigella,
- certain strains of Escherichia coli, and
- certain species of Yersinia.

All intestinal tract infections are transmitted by the fecal-oral route.

There are two species of Salmonella, Salmonella enterica and Salmonella bongori. Any infection caused by Salmonella is called a salmonellosis. Non-typhoidal Salmonella accounts for an estimated 520 cases per 100,000 population (approximately 1,600,000 cases) per year in the U.S. and at least 500 die. Since many different animals carry Salmonella in their intestinal tract, people usually become infected from ingesting improperly refrigerated, uncooked or undercooked poultry, eggs, meat, dairy products, vegetables, or fruit contaminated with animal feces.

Enteritis is the most common form of salmonellosis. Symptoms generally appear 6-48 hours after ingestion of the bacteria and include vomiting, nausea, non-bloody diarrhea, fever, abdominal cramps, myalgias, and headache. Symptoms generally last from 2 days to 1 week followed by spontaneous recovery. All species of Salmonella can cause bacteremia but S. enterica serotype Typhi, isolated only from humans, frequently disseminates into the blood causing a severe form of salmonellosis called typhoid fever. About 400 cases of typhoid fever occur each year in the U.S. but approximately 75% of these are acquired while traveling internationally.

Salmonella serotyping is a subtyping method of identification based on the identification of distinct cell wall, flagellar, and capsular antigens with known antiserum, as will be discussed in Lab 17. Salmonella serotypes Enteritidis and Typhimurium are the two most common serotypes in the United States, accounting for approximately 35 to 40% of all infections confirmed by laboratory culture. As mentioned above, S. enterica serotype Typhi is responsible for typhoid fever.

Any Shigella infection is called a shigellosis. Unlike Salmonella, which can infect many different animals, Shigella only infects humans and other higher primates. There are approximately 14,000 laboratory cases of shigellosis a year reported in the US with an estimated 450,000 total cases and 70 deaths.

Shigellosis frequently starts with a watery diarrhea, fever, and abdominal cramps but may progress to dysentery.
with scant stools containing blood, pus, and mucus. The incubation period is 1-3 days. Initial profuse watery diarrhea typically appears first as a result of enterotoxin. Within 1-2 days this progresses to abdominal cramps, with or without bloody stool. Classic shigellosis presents itself as lower abdominal cramps and stool abundant with blood and pus develops as the *Shigella* invade the mucosa of the colon.

*Escherichia coli* is one of the dominant normal flora in the intestinal tract of humans and animals. Some strains, however, can cause infections of the intestines while others are capable of causing infections outside the intestines. Extraintestinal pathogenic *E. coli* cause such opportunistic infections as urinary tract infections, wound infections, and septicemia and will be discussed in greater detail below. Intestinal or diarrheagenic *E. coli* cause infections of the intestinal tract. Diarrheagenic *E. coli* include:

- **Enterotoxigenic *E. coli*** (ETEC) produce enterotoxins that cause the loss of sodium ions and water from the small intestines resulting in a watery diarrhea. It is an important cause of diarrhea in impoverished countries. Over half of all travelers' diarrhea is due to ETEC; almost 80,000 cases a year in the U.S.
- **Enteropathogenic *E. coli*** (EPEC) causes an endemic diarrhea in impoverished countries, especially in infants younger than 6 months of age. The bacterium disrupts the normal microvilli on the epithelial cells of the small intestines resulting in malabsorption and diarrhea. They do not produce enterotoxin or shiga toxin and are not invasive. It is rare in industrialized countries.
- **Enteroaggregative *E. coli*** (EAEC) is a cause of endemic diarrhea in children in impoverished countries and industrialized countries. It is also responsible for a persistent diarrhea in people infected with HIV. It probably causes diarrhea by adhering to mucus epithelial cells of the small intestines and interfering with their function.
- **Enteroinvasive *E. coli*** (EIEC) invade and kill epithelial cells of the colon usually causing a watery diarrhea but sometimes progressing to a dysentery-type syndrome with blood in the stool. It occurs mostly in impoverished countries and is rare in industrialized countries.
- **Enterohemorrhagic *E. coli*** (EHEC), such as *E. coli* 0157:H7, produce a shiga toxin that kills epithelial cells of the colon causing hemorrhagic colitis, a bloody diarrhea. In rare cases, the shiga toxin enters the blood and is carried to the kidneys where, usually in children, it damages vascular cells and causes hemolytic uremic syndrome. *E. coli* 0157:H7 is thought to cause more than 20,000 infections and up to 250 deaths per year in the U.S.

Several species of *Yersinia*, such as *Y. enterocolitica* and *Y. pseudotuberculosis* are also causes of diarrheal disease.

Many other genera of the family *Enterobacteriaceae* are normal microbiota of the intestinal tract and are considered opportunistic pathogens. The most common genera of *Enterobacteriaceae* causing opportunistic infections in humans are:

- *Escherichia coli*,
- *Proteus*,
- *Enterobacter*,
- *Klebsiella*,
- *Citrobacter*, and
- *Serratia*.

They act as opportunistic pathogens when they are introduced into body locations where they are not normally found, especially if the host is debilitated or immunosuppressed. They all cause the same types of opportunistic infections, namely:
• urinary tract infections,
• wound infections,
• pneumonia, and
• septicemia.

These normal flora Gram-negative bacilli, along with Gram-positive bacteria such as Enterococcus species (see Lab 14) and Staphylococcus species (see Lab 15), are among the most common causes of healthcare-associated infections (formerly called nosocomial infections).

According to the Centers for Disease Control and Prevention (CDC) Healthcare-associated infection's website, "In American hospitals alone, healthcare-associated infections account for an estimated 1.7 million infections and 99,000 associated deaths each year. Of these infections:

• 32 percent of all healthcare-associated infection are urinary tract infections (UTIs)
• 22 percent are surgical site infections
• 15 percent are pneumonia (lung infections)
• 14 percent are bloodstream infections"

Most patients who have healthcare-associated infections are predisposed to infection because of invasive supportive measures such as urinary catheters, intravascular lines, and endotracheal intubation.

By far, the most common Gram-negative bacterium causing nosocomial infections is Escherichia coli. E. coli causes between 70 and 90% of both upper and lower urinary tract infections (UTIs). It is also a frequent cause of abdominal wound infections and septicemia. Depending on the facility, E. coli is responsible for between 12% and 50% of all healthcare-associated infections.

However, according to a 2008 study, Enterobacteriaceae other than E. coli were responsible for 7 of the 10 most common Gram-negative organisms isolated from urinary tract, respiratory tract, and bloodstream infections from intensive care unit patients between 2002 and 2008 in the United States. These include Klebsiella pneumoniae (15%), Enterobacter cloacae (9%), Serratia marcescens (6%), Enterobacter aerogenes (4%), Proteus mirabilis (4%), Klebsiella oxytoca (3%), and Citrobacter freundii (2%). Furthermore, the National Healthcare Safety Network reported K. pneumoniae (6%), Enterobacter spp. (5%), and K. oxytoca (2%) among the top 10 most frequently isolated health care-associated infections between the years between 2006 and 2007.

1. Urinary Tract Infections

The most common infection caused by opportunistic Enterobacteriaceae is a urinary tract infection (UTI). UTIs account for more than 8,000,000 physician office visits per year in the U.S and as many as 100,000 hospitalizations. Among the nonhospitalized and nondebilitated population, UTIs are more common in females because of their shorter urethra and the closer proximity between their anus and the urethral opening. (Over 20 percent of women have recurrent UTIs.) However, anyone can become susceptible to urinary infections in the presence of predisposing factors that cause functional and structural abnormalities of the urinary tract. These abnormalities increase the volume of residual urine and interfere with the normal clearance of bacteria by urination. Such factors include
prostate enlargement, sagging uterus, expansion of the uterus during pregnancy, paraplegia, spina bifida, scar tissue formation, and catheterization. Between 35 and 40 percent of all nosocomial infections, about 900,000 per year in the U.S., are UTIs and are usually associated with catheterization.

**E. coli and Staphylococcus saprophyticus** (a Gram-positive staphylococcus that will be discussed in Lab 15) cause around 90 percent of all uncomplicated UTIs. Most of the remaining uncomplicated UTIs are caused by other Gram-negative enterics such as *Proteus mirabilis* and *Klebsiella pneumoniae* or by *Enterococcus faecalis* (a Gram-positive streptococcus that will be discussed in Lab 14). **E. coli is responsible for more than 50 percent of healthcare-associated UTIs.** Other causes of hospital-acquired UTIs include other species of *Enterobacteriaceae* (such as *Proteus*, *Enterobacter*, and *Klebsiella*), *Pseudomonas aeruginosa* (discussed below), *Enterococcus* species (discussed in lab 14), *Staphylococcus saprophyticus* (discussed in Lab 15), and the yeast *Candida* (discussed in lab 9).

The traditional laboratory culture standard for a UTI has been the presence of more than 100,000 CFUs (colony-forming units; see Lab 4) per milliliter (ml) of midstream urine, or any CFUs from a catheter-obtained urine sample. More recently, this has been modified and counts of as few as 1000 colonies of a single type per ml or as little as 100 coliforms per ml are now considered as indicating a UTI.

2. Wound Infections

**Wound infections** are due to fecal contamination of external wounds or a result of wounds that cause trauma to the intestinal tract, such as surgical wounds, gunshot wounds, and knife wounds. In the latter case, fecal bacteria get out of the intestinal tract and into the peritoneal cavity causing peritonitis and formation of abscesses on the organs found in the peritoneal cavity.

3. Pneumonia

Although they sometimes cause pneumonia, the *Enterobacteriaceae* account for less than 5% of the bacterial pneumonias requiring hospitalization.

4. Bloodstream Infections

**Gram-negative septicemia** is a result of these opportunistic Gram-negative bacteria getting into the blood. They are usually introduced into the blood from some other infection site, such as an infected kidney, wound, or lung. Looking at patients that develop septic shock:

- Lower respiratory tract infections are the source in about 25% of patients.
- Urinary tract infections are the source in about 25% of patients.
- Soft tissue infections are the source in about 15% of patients.
- Gastrointestinal infections are the source in about 15% of patients.
- Reproductive tract infections are the source in about 10% of patients.
- Foreign bodies (intravascular lines, implanted surgical devices, etc.) are the source in about 5% of patients.

There are approximately 750,000 cases of septicemia per year in the U.S. and 200,000 cases of septic shock. Septic shock results in approximately 100,000 deaths per year in the U.S. Approximately 45 percent of the cases of
Septicemia are due to Gram-negative bacteria. *Klebsiella*, *Proteus*, *Enterobacter*, *Serratia*, and *E. coli*, are all common *Enterobacteriaceae* causing septicemia. (Another 45 percent are a result of Gram-positive bacteria (see Labs 14 and 15) and 10 percent are due to fungi, mainly the yeast *Candida* (see Lab 9).

In the outer membrane of the Gram-negative cell wall, the lipid A moiety of the lipopolysaccharide (LPS) functions as an endotoxin. Endotoxin indirectly harms the body when massive amounts are released during severe Gram-negative infections. This, in turn, causes an excessive cytokine response.

1. The LPS released from the outer membrane of the Gram-negative cell wall first binds to a LPS-binding protein circulating in the blood and this complex, in turn, binds to a receptor molecule (CD14) found on the surface of body defense cells called macrophages (see Fig. 5) located in most tissues and organs of the body.

2. This is thought to promote the ability of the toll-like receptor TLR-4 to respond to the LPS, triggering the macrophages to release various defense regulatory chemicals called cytokines, including tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8), and platelet-activating factor (PAF). The cytokines then bind to cytokine receptors on target cells and initiate inflammation and activate both the complement pathways and the coagulation pathway (see Fig. 5).

3. The complex of LPS and LPS binding protein can also attach to molecules called CD14 on the surfaces of phagocytic white blood cells called neutrophils causing them to release proteases and toxic oxygen radicals for extracellular killing. Chemokines such as interleukin-8 (IL-8) also stimulate extracellular killing. In addition, LPS and cytokines stimulate the synthesis of a vasodilator called nitric oxide.

During minor local infections with few bacteria present, low levels of LPS are released leading to moderate cytokine production by the monocytes and macrophages and in general, promoting body defense by stimulating inflammation and moderate fever, breaking down energy reserves to supply energy for defense, activating the complement pathway and the coagulation pathway, and generally stimulating immune responses (see Fig. 5). Also as a result of these cytokines, circulating phagocytic white blood cells such as neutrophils and monocytes stick to the walls of capillaries, squeeze out and enter the tissue, a process termed diapedesis. The phagocytic white blood cells such as neutrophils then kill the invading microbes with their proteases and toxic oxygen radicals.

However, during severe systemic infections with large numbers of bacteria present, high levels of LPS are
released resulting in excessive cytokine production by the monocytes and macrophages and this can harm the body (see Fig. 6). In addition, neutrophils start releasing their proteases and toxic oxygen radicals that kill not only the bacteria, but the surrounding tissue as well. Harmful effects include high fever, hypotension, tissue destruction, wasting, acute respiratory distress syndrome (ARDS), disseminated intravascular coagulation (DIC), and damage to the vascular endothelium resulting in shock, multiple system organ failure (MOSF), and often death.

This excessive inflammatory response is referred to as Systemic Inflammatory Response Syndrome or SIRS. Death is a result of what is called the shock cascade. The sequence of events is as follows:

- Neutrophil-induced damage to the capillaries, as well as prolonged vasodilation, results in blood and plasma leaving the bloodstream and entering the surrounding tissue. This can lead to a decreased volume of circulating blood (hypovolemia).
- Prolonged vasodilation also leads to decreased vascular resistance within blood vessels while high levels of TNF inhibit vascular smooth muscle tone and myocardial contractility. This results in a marked hypotension.
- Activation of the blood coagulation pathway can cause clots called microthrombi to form within the blood vessels throughout the body causing disseminated intravascular coagulation (DIC).
- Increased capillary permeability as a result of vasodilation in the lungs, as well as neutrophil-induced injury to capillaries in the alveoli, lead to acute inflammation, pulmonary edema, and loss of gas exchange in the lungs (acute respiratory distress syndrome or ARDS). As a result, the blood does not become oxygenated.
- Hypotension, hypovolemia, ARDS, and DIC result in marked hypoperfusion.
- Hypoperfusion in the liver can result in a drop in blood glucose level from liver dysfunction.
- Hypoperfusion leads to acidosis and the wrong pH for enzymes involved in cellular metabolism resulting in cell death.
- Hypoperfusion also can lead to cardiac failure.

Collectively, this can result in:

- end-organ ischemia: a restriction in blood supply that results in damage or dysfunction of tissues or organs,
- multiple system organ failure (MSOF),
- death.

More information on SIRS and septic shock from your Lecture E-text

Both pili and surface proteins in the Gram-negative cell wall function as adhesins, allowing the bacterium to adhere intimately to host cells and other surfaces in order to colonize and resist flushing. Some Gram-negative bacteria also produce invasins, allowing some bacteria to invade host cells. Motility, capsules, biofilm formation, and exotoxins also play a role in the virulence of some Enterobacteriaceae.

For further information on virulence factors associated with various Enterobacteriaceae, see the following Learning Objects in your Lecture Guide:

- **Endotoxin: Unit 3, Section C1b**
- **The Ability to Adhere to Host Cells: Pili and Adhesins: Unit 3, Section B2**
The Ability to Invade Host Cells; Unit 3, Section B3
The Ability to Contact Host Cells; Unit 3, Section B1
The Ability to Resist Phagocytic Engulfment; Unit 3, Section B5b
The Ability to Resist Phagocytic Destruction and Serum Lysis; Unit 3, Section B5c
A-B Toxins; Unit 3, Section C2b
Toxins that Damage Cell Membranes; Unit 3, Section C2c

Many of the Enterobacteriaceae also possess R (resistance) plasmids. These plasmids are small pieces of circular non-chromosomal DNA that may code for multiple antibiotic resistance. In addition, the plasmid may code for a sex pilus, enabling the bacterium to pass R plasmids to other bacteria by conjugation. Between 50 and 60 percent of the bacteria causing healthcare-associated infections are antibiotic resistant.

For further information on bacterial resistance to antibiotics, see the following Learning Object in your Lecture E-Text:

- How Bacteria Resist Our Control Agents; Unit 2, Section IIC
- Horizontal Gene Transfer in Bacteria; Unit 2, Section IA

The identification of lactose-fermenting Gram-negative rods belonging to the bacterial family Enterobacteriaceae (bacteria commonly referred to as coliforms) in water is often used to determine if water has been fecally contaminated and, therefore, may contain disease-causing pathogens transmitted by the fecal-oral route. The procedure for this is given in Appendix E.

B. PSEUDOMONAS AND OTHER NON-FERMENTATIVE GRAM-NEGATIVE BACILLI

Non-fermentative Gram-negative bacilli refer to Gram-negative rods or coccobacilli that cannot ferment sugars. The non-fermentative Gram-negative bacilli are often normal inhabitants of soil and water. They may cause human infections when they colonize immunosuppressed individuals or gain access to the body through trauma. However, less than one-fifth of the Gram-negative bacilli isolated from clinical specimens are non-fermentative bacilli. By far, the most common Gram-negative, non-fermentative rod that causes human infections is Pseudomonas aeruginosa. Pseudomonas belongs to the family Pseudomonadaceae in the order Pseudomonadales in the class Gammaproteobacter of the phylum Proteobacter.

Pseudomonas aeruginosa is also an opportunistic pathogen. It is a common cause of nosocomial infections and can be found growing in a large variety of environmental locations. In the hospital environment, for example, it has been isolated from drains, sinks, faucets, water from cut flowers, cleaning solutions, medicines, and even disinfectant soap solutions. It is especially dangerous to the debilitated or immunocompromised patient.
Like the opportunistic Enterobacteriaceae, Pseudomonas is a Gram-negative rod, it is frequently found in small amounts in the feces, and it causes similar opportunistic infections: urinary tract infections, wound infections, pneumonia, and septicemia. P. aeruginosa is the fourth most commonly isolated nosocomial pathogen, accounting for 10% of all hospital-acquired infections. P. aeruginosa is responsible for 12 percent of hospital-acquired urinary tract infections, 16 percent of nosocomial pneumonia cases, and 10 percent of the cases of septicemia. In addition, P. aeruginosa is a significant cause of burn infections with a 60 percent mortality rate. It also colonizes and chronically infects the lungs of people with cystic fibrosis. Like other opportunistic Gram-negative bacilli, Pseudomonas aeruginosa also releases endotoxin and frequently possesses R-plasmids. A number of other species of Pseudomonas have also been found to cause human infections.

- Electron micrograph of Pseudomonas aeruginosa colonizing a vascular catheter.

For further information on virulence factors associated with Pseudomonas, see the following Learning Objects in your Lecture Guide:

- Endotoxin; Unit 3, Section C1b
- The Ability to Adhere to Host Cells: Pili and Adhesins; Unit 3, Section B2
- The Ability to Invade Host Cells; Unit 3, Section B3
- The Ability to Contact Host Cells; Unit 3, Section B1
- The Ability to Resist Phagocytic Engulfment; Unit 3, Section B5b
- The Ability to Resist Phagocytic Destruction and Serum Lysis; Unit 3, Section B5c
- A-B Toxins; Unit 3, Section C2b
- Toxins that Damage Cell Membranes; Unit 3, Section C2c

Other non-fermentative Gram-negative bacilli that are sometimes opportunistic pathogens in humans include Acinetobacter, Aeromonas, Alcaligenes, Eikenella, Flavobacterium, and Moraxella.

Acinetobacter has become a frequent cause of nosocomial wound infections, pneumonia, and septicemia. The bacterium has become well known as a cause of infections among veterans of the wars in Iraq and Afghanistan and is becoming a growing cause of nosocomial infections in the U.S. Acinetobacter is thought to have been contracted in field hospitals in Iraq and Afghanistan and subsequently carried to veteran's hospitals in the U.S. Because most species are multiple antibiotic resistant, it is often difficult to treat. Acinetobacter is commonly found in soil and water, as well as on the skin of healthy people, especially healthcare personnel. Although there are numerous species of Acinetobacter that can cause human disease, Acinetobacter baumannii accounts for about 80% of reported infections.

- Diseases and Organisms in Healthcare Settings; from CDC

Medscape articles on infections associated with organisms mentioned in this lab exercise. Registration to access this website is free.
• Salmonellosis
• Typhoid fever
• Shigellosis
• *Escherichia coli*
• *Proteus* species
• *Klebsiella* species
• *Enterobacter* species
• *Serratia* species
• *Yersinia enterocolitica*
• *Yersinia pseudotuberculosis*
• *Acinetobacter baumannii*
• *Pseudomonas aeruginosa*
• Urinary tract infections
• Wound infections
• Community-acquired pneumonia
• Nosocomial pneumonia
• Sepsis
• Septic shock

**SCENERIOS FOR TODAY’S LAB**

Students will be assigned either **Case Study 1A or 1B** to do today. **All students will do Case Study 2** as part of the results next time.

**Case Study #1A**

A 66 year old female with a history of recurring urinary tract infections and multiple antibiotic therapies presents with frequency and urgency of urination, dysuria, suprapubic discomfort, lower back pain, and a temperature of 99.2°F. A complete blood count (CBC) shows leukocytosis with a left shift. A urine dipstick shows a positive leukocyte esterase test, a positive nitrite test, 30mg of protein per deciliter, and red blood cells in the urine.

Assume that your unknown is a urine culture from this person.

**Case Study #1B**

A 72 year old female who is diabetic and a smoker was admitted to the hospital with a leg wound that is not healing. She appears confused and anxious, has a temperature of 102°F, a heart rate of 101 beats per minute, a respiration rate of 29 breaths per minute, a blood pressure of 94/32 mm Hg, a urine output of only 110 cc for the last 8 hours, and a total white blood cell count of 2300/µL. A blood culture is taken.
Assume that your unknown is a blood culture from this person.

**CAUTION: TREAT EACH UNKNOWN AS A PATHOGEN!** Inform your instructor of any spills or accidents. **WASH AND SANITIZE YOUR HANDS WELL** before leaving the lab.

### MATERIALS

Taxo N® disk, alcohol, dropper bottle of distilled water, swab, and **either** a plate of MacConkey agar or a plate of Cetrimide agar, and an EnteroPluri-Test

### PROCEDURE (to be done in groups of 3)

[Keep in mind that organisms other than the *Enterobacteriaceae* and *Pseudomonas* can cause these infections, so in a real clinical situation other lab tests and cultures for bacteria other than those upon which this lab is based would also be done.]

1. Perform a **Gram stain** on your unknown. Remember that the concentration of bacteria on slides prepared from taking bacteria off a petri plate tend to be much greater than those prepared by taking bacteria out of a broth culture, so be **careful not to under decolorize**. Continue decolorizing until the purple just stops flowing off of the bacterial smear, then wash with water.

   - Record the results of your Gram stain in the Gram stain section of Lab 13.

   - Instructions for the Gram Stain from Lab 6.

   - Instructions for Focusing a Microscope from Lab 1.

2. If you have a Gram-negative bacillus, determine if it is a **fermentative** Gram-negative bacillus like most *Enterobacteriaceae* or a **non-fermentative** Gram-negative bacillus such as *Pseudomonas* by performing an **oxidase test** as follows:

   a. Using alcohol-flamed forceps, remove a Taxo-N® disc and moisten it with a drop of sterile distilled water.

   b. Place the moistened disc on the colonies of the culture of your unknown.

   c. Using a sterile swab, scrape off some of the colonies and spread them on the Taxo-N® disc.

   In the **immediate test**, oxidase-positive reactions will turn a **rose color within 30 seconds** (see Fig. 10). Oxidase-negative will not turn a rose color (see Fig. 9). This reaction only lasts a couple of minutes. In the **delayed test**, oxidase-positive colonies within 10 mm of the Taxo-N® disc will turn **black within 20 minutes and will remain black** (see Fig. 11). If the bacterium is oxidase-negative, the growth around the disc will not turn black (see Fig. 12).

   - Record your oxidase test results in the Oxidase test section of Lab 13.

3. If your unknown is **oxidase-negative**, indicating a fermentative Gram-negative bacillus, do the following inoculations:
a. Streak your unknown for isolation on a plate of MacConkey agar, a selective medium used for the isolation of non-fastidious Gram-negative rods and particularly members of the family Enterobacteriaceae, using one of the two streaking patterns illustrated in Fig. 4 and Fig. 5. Incubate upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section.

b. Inoculate an EnteroPluri-Test as follows:

1. Remove both caps of the EnteroPluri-Test and with the straight end of the inoculating wire, pick off the equivalent of a colony from your unknown plate. A visible inoculum should be seen on the tip and side of the wire.

2. Inoculate the EnteroPluri-Test by grasping the bent-end of the inoculating wire, twisting it, and withdrawing the wire through all 12 compartments using a turning motion.

3. Reinsert the wire into the tube (use a turning motion) through all 12 compartments until the notch on the wire is aligned with the opening of the tube. (The tip of the wire should be seen in the citrate compartment.) Break the wire at the notch by bending. Do not discard the wire yet.

4. Using the broken off part of the wire, punch holes through the cellophane which covers the air inlets located on the rounded side of the last 8 compartments. Your instructor will show you their correct location. Discard the broken off wire in the disinfectant container.

5. Replace both caps and incubate the EnteroPluri-Test on its flat surface at 36°C - 37°C for 18-24 hours.

4. If your unknown is oxidase-positive, indicating a non-fermentative Gram-negative bacillus, do the following inoculation:

   a. Streak your unknown for isolation on a plate of Cetrimide agar, a selective and differential medium for Pseudomonas, using one of the two streaking patterns illustrated in Fig. 4 and Fig. 5. Incubate upside down and stacked in the petri plate holder on the shelf of the 37°C incubator corresponding to your lab section.

   Note that MacConkey agar can also be used to isolate Pseudomonas but we are using the Cetrimide agar today because it enables us to detect the production of the blue to green water-soluble pigment by Pseudomonas aeruginosa, as well as the production of fluorescein.

You will also inoculate an EnteroPluri-Test for practice only, but keep in mind that the EnteroPluri-Test is used
to identify *Enterobacteriaceae*, not *Pseudomonas*.

Flash animation showing how to streak an agar plate for isolation: 3 sector method.

html5 version of animation for iPad showing how to streak an agar plate for isolation: 3 sector method.

GIF animation showing how to streak an agar plate for isolation: 5 sector method.

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**Case Study #2**

After receiving a baby chicken for Easter, a 7 year old boy is taken to the emergency room with symptoms of vomiting, nausea, non-bloody diarrhea, abdominal cramps, and a temperature of 100°F. A complete blood count (CBC) shows the WBC count to be within the reference range.

This XLD agar plate and this EnteroPluri-Test are from a stool culture from this patient.

**CAUTION: TREAT THE UNKNOWN AS A PATHOGEN!**. Inform your instructor of any spills or accidents. **WASH AND SANITIZE YOUR HANDS WELL** before leaving the lab.

**MATERIALS**

Demonstration XLD agar plate and EnteroPluri-Test

**PROCEDURE** (to be done in groups of 3)

1. Observe the following demonstrations **shown in the links directly below** and identify the causative bacterium:
   
   a. An XLD agar plate, a selective medium used for isolating and differentiating Gram-negative enteric bacteria, especially intestinal pathogens such as *Salmonella* and *Shigella*.
   
   b. The EnteroPluri-Test.

2. Record your results in the Results section of Lab 13.

**C. Lab Tests Used as Part of Today's Lab**

To isolate *Enterobacteriaceae* and *Pseudomonas*, specimens from the infected site are plated out on any one of a large number of **selective and differential media** such as EMB agar, Endo agar, Deoxycholate agar, MacConkey agar, Hektoen Enteric agar, and XLD agar. We will look at three of these.

1. MacConkey Agar
MacConkey agar is a selective medium used for the isolation of non-fastidious Gram-negative rods, particularly members of the family Enterobacteriaceae and the genus Pseudomonas, and the differentiation of lactose fermenting from lactose non-fermenting Gram-negative bacilli. MacConkey agar contains the dye crystal violet well as bile salts that inhibit the growth of most Gram-positive bacteria but do not affect the growth of most Gram-negatives (see Fig. 6).

If the Gram-negative bacterium ferments the sugar lactose in the medium, the acid end products lower the pH of the medium. The neutral red in the agar turns red in color once the pH drops below 6.8. As the pH drops, the neutral red is absorbed by the bacteria, causing the colonies to appear bright pink to red.

- **Strong fermentation of lactose with high levels of acid production** by the bacteria causes the colonies and confluent growth to appear bright pink to red. The resulting acid, at high enough concentrations, can also causes the bile salts in the medium to precipitate out of solution causing a pink precipitate (cloudiness) to appear in the agar surrounding the growth (see Fig. 13).
- **Weak fermentation of lactose** by the bacteria causes the colonies and confluent growth to appear pink to red, but without the precipitation of bile salts there is no pink halo around the growth (see Fig. 15).
- **If the bacteria do not ferment lactose**, the colonies and confluent growth appear colorless and the agar surrounding the bacteria remains relatively transparent (see Fig. 17).

Typical colony morphology of our strains of Enterobacteriaceae and Pseudomonas aeruginosa on MacConkey agar is as follows:

1. *Escherichia coli*: colonies and confluent growth appear bright pink to red and surrounded by a pink precipitate (cloudiness) in the agar surrounding the growth (see Fig. 13). Strong fermentation of lactose.

2. *Klebsiella pneumoniae*: colonies and confluent growth appear bright pink to red but are not surrounded by a pink precipitate (cloudiness) in the agar surrounding the growth (see Fig. 14). Weak fermentation of lactose.

3. *Enterobacter aerogenes*: colonies and confluent growth appear bright pink to red but are not surrounded by a pink precipitate (cloudiness) in the agar surrounding the growth (see Fig. 15). Weak fermentation of lactose.

4. *Enterobacter cloacae*: colonies and confluent growth appear bright pink to red but are not surrounded by a pink precipitate (cloudiness) in the agar surrounding the growth (see Fig. 16). Weak fermentation of lactose.

5. *Proteus mirabilis*: colorless colonies; agar relatively transparent (see Fig. 17). No fermentation of lactose.

6. *Proteus vulgaris*: colorless colonies; agar relatively transparent (see Fig. 18). No fermentation of lactose.

7. *Serratia marcescens*: colorless colonies; agar relatively transparent (see Fig. 19). No fermentation of lactose.

8. *Pseudomonas aeruginosa*: colorless colonies; agar relatively transparent (see Fig. 20).

9. *Salmonella enterica*: colorless colonies; agar relatively transparent (see Fig. 21). No fermentation of lactose.

2. XLD Agar

Xylose Lysine Desoxycholate (XLD) agar is used for isolating and differentiating Gram-negative enteric...
bacteria, especially intestinal pathogens such as *Salmonella* and *Shigella*. XLD agar contains sodium desoxycholate, which inhibits the growth of Gram-positive bacteria but permits the growth of Gram-negatives. It also contains the sugars lactose and sucrose, the amino acid L-lysine, sodium thiosulfate, and the pH indicator phenol red. Results can be interpreted as follows:

- If the Gram-negative bacterium ferments lactose and/or sucrose, acid end products will be produced and cause the colonies and the phenol red in the agar around the colonies to turn yellow (see Fig. 16).
- If lactose and sucrose are not fermented by the bacterium but the amino acid lysine is decarboxylated, ammonia, an alkaline end product will cause the phenol red in the agar around the colonies to turn a deeper red (see Fig. 17).
- Sometimes the bacterium ferments the sugars producing acid end products and breaks down lysine producing alkaline end products. In this case some of the colonies and part of the agar turns yellow and some of the colonies and part of the agar turns a deeper red (see Fig. 18).
- If hydrogen sulfide is produced by the bacterium as a result of thiosulfate reduction, part or all of the colony will appear black (see Fig. 19). Well-isolated colonies are usually needed for good results.

Typical colony morphology on XLD agar is as follows:

1. *Escherichia coli*: flat yellow colonies; some strains may be inhibited.
2. *Enterobacter* and *Klebsiella*: mucoid yellow colonies.
3. *Proteus*: red to yellow colonies; may have black centers.
4. *Salmonella*: usually red colonies with black centers.
5. *Shigella*, *Serratia*, and *Pseudomonas*: red colonies without black centers

Keep in mind, however, that some species and subspecies do not show typical reactions.

3. Cetrimide Agar (*Pseudomonas* P agar)

Cetrimide agar contains the chemical cetrimide (cetyl trimethylammonium bromide) for the selective inhibition of most bacteria other than *Pseudomonas*. The medium also stimulates *Pseudomonas aeruginosa* to produce a number of water soluble iron chelators, including pyoverdin and pyocyanin. The green water soluble color characteristic of *Pseudomonas aeruginosa* is created when the yellow-green or yellow-brown fluorescent pyoverdin combines with the blue water-soluble pyocyanin (see Fig. 20). The fluorescent pyoverdin will typically fluoresce when the plate is placed under a short wavelength ultraviolet light (see Fig. 21). After a few minutes at room temperature, the plate loses its fluorescence. The fluorescence, however, can be restored by placing the plate back at 37°C for several minutes.

4. Oxidase Test

In this lab a Taxo N® disc is used to perform the oxidase test. The oxidase test is based on the bacterial production of an oxidase enzyme. *Cytochrome oxidase*, in the presence of oxygen, oxidizes the para-amino dimethylanaline oxidase test reagent in a Taxo-N® disc.
• In the **immediate test**, oxidase-positive reactions will turn a **rose color within 30 seconds** (see Fig. 5). Oxidase-negative will not turn a rose color (see Fig. 6). This reaction only lasts a couple of minutes.

• In the **delayed test**, oxidase-positive colonies within 10 mm of the Taxo-N® disc will **turn black within 20 minutes and will remain black** (see Fig. 7). If the bacterium is oxidase-negative, the growth around the disc will not turn black (see Fig. 8).

*Pseudomonas aeruginosa* and most other non-fermentative, Gram-negative bacilli are oxidase-positive; with the exception of the genus *Plesiomonas*, the *Enterobacteriaceae* are oxidase-negative.

5. Pigment production in *Pseudomonas aeruginosa*

The green water soluble color characteristic of *Pseudomonas aeruginosa* is created when the yellow-green or yellow-brown fluorescent pyoverdin combines with the blue water-soluble pyocyanin (see Fig. 20). The fluorescent pyoverdin will typically fluoresce when the plate is placed under a short wavelength ultraviolet light (see Fig. 21). After a few minutes at room temperature, the plate loses its fluorescence. The fluorescence, however, can be restored by placing the plate back at 37°C for several minutes. None of the *Enterobacteriaceae* produces pigment at 37°C.

6. Odor

Most of the *Enterobacteriaceae* have a rather foul smell; *Pseudomonas aeruginosa* produces a characteristic **fruity or grape juice-like aroma** due to production of an aromatic compound called aminoacetophenone.

7. The EnteroPluri-Test

A number of techniques can be used for the identification of specific species and subspecies of *Enterobacteriaceae*. Speciation is important because it provides data regarding patterns of susceptibility to antimicrobial agents and changes that occur over a period of time. It is also essential for epidemiological studies such as determination of nosocomial infections and their spread.

In an effort to simplify the speciation of the *Enterobacteriaceae* and reduce the amount of prepared media and incubation space needed by the clinical lab, a number of self-contained **multi-test systems** have been commercially marketed. Some of these multi-test systems have been combined with a computer-prepared manual to provide identification based on the overall **probability of occurrence** for each of the biochemical reactions. In this way, a large number of biochemical tests can economically be performed in a short period of time, and the results can be accurately interpreted with relative ease and assurance.

**The EnteroPluri-Test** (see Fig. 22) is a self-contained, compartmented plastic tube containing 12 different agars (enabling the performance of a total of 15 standard biochemical tests) and an enclosed inoculating wire. After inoculation and incubation, the resulting combination of reactions, together with a Computer Coding and Identification System (CCIS), allows for easy identification. The various biochemical reactions of the EnteroPluri-Test and their correct interpretation are discussed below. Although it is designed to identify members of the bacterial family *Enterobacteriaceae*, it will sometimes also identify common biotypes of *Pseudomonas* and other non-fermentative Gram-negative bacilli. It does not identify *Pseudomonas aeruginosa*. 
IDENTIFYING MEMBERS OF THE ENTEROBACTERIACEAE WITH THE ENTEROPLURI-TEST

The EnteroPluri-Test contains 12 different agars that can be used to carry out 15 standard biochemical tests (see Fig. 22). Interpret the results of your EnteroPluri-Test using the instructions below and record them on the EnteroPluri-Test table on your Results page. For more detail on the 15 biochemical tests in the EnteroPluri-Test, see Table 13A.

1. Interpret the results of glucose fermentation in compartment 1.
   - Any yellow = +; red = -
   - If positive, circle the number 4 under glucose on your Results page.

2. Interpret the results of gas production also in compartment 1.
   - White wax lifted from the yellow agar = +; wax not lifted from agar = -
   - If positive, circle the number 2 under gas on your Results page.

3. Interpret the results of lysine decarboxylase in compartment 2.
   - Any violet = +; yellow = -
   - If positive, circle the number 1 under lysine on your Results page.

4. Interpret the results of ornithine decarboxylase in compartment 3.
   - Any violet = +; yellow = -
   - If positive, circle the number 4 under ornithine on your Results page.

5. Interpret the results of H₂S production in compartment 4.
   - Black/brown = +; beige = - (The black may fade or revert back to negative if the EnteroPluri-Test is read after 24 hours of incubation.)
   - If positive, circle the number 2 under H₂S on your Results page.

6. Indole production also in compartment 4. Do not interpret the indole test at this time. Add Kovac's reagent only after all other tests have been read (see step 16 below).

7. Interpret the results of adonitol fermentation in compartment 5.
   - Any yellow = +; red = -
   - If positive, circle the number 4 under adonitol on your Results page.

8. Interpret the results of lactose fermentation in compartment 6.
   - Any yellow = +; red = -
   - If positive, circle the number 2 under lactose on your Results page.

9. Interpret the results of arabinose fermentation in compartment 7.
10. Interpret the results of sorbitol fermentation in compartment 8.
   • Any yellow = +; red = -
   • If positive, circle the number 4 under sorbitol on your Results page.

11. Voges-Praskauer (VP) test in compartment 9. Do not interpret the VP test at this time. Add the reagents alpha-naphtol and potassium hydroxide (KOH) only after all other tests have been read (see step 17 below).

12. Interpret the results of dulcitol fermentation in compartment 10.
   • Yellow = +; green or dark brown = -
   • If positive, circle the number 1 under dulcitol on your Results page.

13. Interpret the results of PA deaminase also in compartment 10.
   • Dark brown= +; green or yellow= -
   • If positive, circle the number 4 under PA on your Results page.

14. Interpret the results of urea hydrolysis in compartment 11.
   • Pink, red or purple = +; beige = -
   • If positive, circle the number 2 under urea on your Results page.

15. Interpret the results of citrate utilization in compartment 12.
   • Any blue = +; green = -
   • If positive, circle the number 1 under citrate on your Results page.

16. Your instructor will add 2-3 drops of Kovac's reagent to the indole test compartment.
   • Pink/red = +; yellow = -
   • If positive, circle the number 1 under indole on your Results page.

17. Your instructor will add 3 drops of alpha-naphtol reagent and 2 drops of potassium hydroxide (KOH) to the VP test compartment.
   • Red = +; colorless = -
   • If positive, circle the number 2 under VP on your Results page.

18. Add all the positive test number values in each bracketed section and enter each sum in its code box on the EnteropLuri-Test chart on your Results page.

19. The 5 digit number is the CODICE number. Look that number up in the Codebook and identify your unknown. (Should more than one organism be listed, the confirmatory tests indicated in the CCIS would normally then have to be performed. In addition, an identification of Salmonella or Shigella would usually be confirmed by...
If there are any problems, consult your instructor.

<table>
<thead>
<tr>
<th>Test</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
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<tr>
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<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Gas</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Ornithine</td>
<td>H₂S</td>
<td>Indole</td>
<td>Adonitol</td>
<td>Lactose</td>
</tr>
<tr>
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<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Lactose</td>
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<td>1</td>
<td>4</td>
</tr>
<tr>
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<td>VP</td>
<td>Sorbitol</td>
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<td>Dulcitol</td>
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CODICE NUMBER:

8. The Complete Blood Count (CBC) Test

See Appendix C.

9. Urinalysis (The Dipstick Tests)

See Appendix D.

10. SIRS and Sepsis

See Appendix F

Contributors and Attributions

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