CHARACTERIZATION OF INDIGENOUS BACTERIA AND PATHOGENS

All plants and animals are natural habitats for bacteria. The naturally occurring, and generally nonpathogenic bacteria of the body are called **indigenous bacteria**. The major body sites of animals colonized by indigenous bacteria include the skin, mouth, upper and lower respiratory tract, gastrointestinal system, and genitourinary tract–essentially all regions exposed to the outside environment. Each of these sites is a microhabitat supporting a unique community of bacteria. Most of the indigenous bacteria are normally nonpathogenic and live in a balanced symbiosis with the host organism. Table 1 identifies some common indigenous bacteria of the mouth and throat of humans.

Under certain circumstances some indigenous bacteria can become pathogenic and are said to be **opportunistic pathogens**. They may infect the body through wounds to the skin, or cause disease when a person's health or immune system are impaired. Some bacteria may be pathogenic if they become displaced to an abnormal site on the body. For example, certain species of *Neisseria* are usually nonpathogenic when present in the nasal or genitourinary tract, but can cause serious infections if introduced around the eye.

Among the common human pathogens are *Staphylococcus* and the streptococci. Many species of these genera are also **commensals** on the body, and successful diagnosis of infection requires distinguishing these from pathogens. Thus, many specialized media and procedures have been developed to identify these organisms quickly and effectively.

Identifying pathogens among the numerous beneficial and commensal bacteria can be quite challenging on several accounts. On one hand, the normal indigenous bacteria greatly out-number the pathogens even when a person is gravely ill (for certain species, one pathogen per 10 ml of blood is sufficient to cause serious septicemia). Furthermore, the pathogens often have fastidious nutritional requirements, necessitating the use of different types of media to detect different types of pathogens. Also, since some bacteria are opportunistic, bacteria that are non-virulent in one person may be virulent in someone else.

Blood Agar

Blood agar is composed of a complex medium base (such as TSA) supplemented with whole sheep erythrocytes. Blood agar is the most widely use medium in clinical labs because it supports the growth of most common indigenous bacteria and many **fastidious** pathogens (organisms that do not grow well on standard media).

One of the most important uses of blood agar is in the detection of **hemolysis**, caused by the release of hemolysin (cell rupturing) toxins. When grown on blood agar the colonies of hemolytic bacteria produce a clear zone in the media around the colony. The clearing of blood agar is due to the hemolytic toxins being released.

Blood Agar	
Trypticase	17 g/L
Phytone	3
NaCl	5
K_2HPO_4	2.5
Glucose	2.5
Agar	1
Sheep blood	5% v/v

The appearance of the zone around a colony depends upon the type of toxin that is released form the cells, and this characteristic is important in the identification of bacteria:

Hemolysis	zone appearance	type of lysis
Alpha (α)	greenish-brown	partial
Beta (β)	clear	complete
Gamma (γ)	no zone	no hemolysis

(γ is the designation for the absence of hemolysis)

Summary of exercise

You will study the characteristics of *Staphylococcus* and *Streptococcus* bacteria that aid in the clinical study of human pathogens.

***** CAUTION *****

In this week's lab exercise you will be examining pathogenic and potentially pathogenic bacteria. YOU WILL BE EXPECTED TO USE THE UTMOST CARE IN HANDLING THESE ORGANISMS.

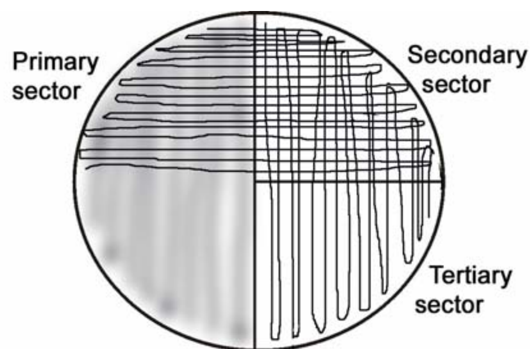
Cultures Cultures: some are provided on plates and broth culture

Enterococcus faecalis (purple cap)
Streptococcus pyogenes (green cap)
Neisseria subflava
Staphylococcus aureus (blue cap)
Your semester unknown
Alcaligenes faecalis (clear cap)

I. Survey of Hemolytic Bacteria in the Mouth

In a clinical lab, microbiologists frequently use the quadrant method (Figure 1; and as demonstrated in class) to streak a clinical specimen. This method has the advantage of allowing a better overview of the diversity of bacteria present (via colony morphology) in the specimen. Isolated colonies also can be selected for further analysis. In this technique, the sample is swabbed over primary sector of the plate, and then an inoculating loop is used to spread bacteria into the secondary and tertiary sectors.

Figure 1. Clinical Streaking Technique



Supplies

2 sterile cotton swabs
2 blood agar plates

Procedure

Each team member should innoculate a blood agar plate from their mouth.

1. Dampen a cotton swab with saliva from the mouth, and then innoculate the primary sector of a blood agar plate.
2. Using your inoculating loop, draw the sample into the secondary and tertiary sectors.
3. Incubate the plates at 37°C for 24 - 48 hours.
4. Record your observations.

II. Identification of Streptococci

Streptococci are non-motile, Gram-positive cocci arranged in short to long chains. Unlike *Staphylococcus*, many *Streptococcus* species have fastidious nutritional needs, and generally do not grow well on standard complex media. For this reason, blood agar is generally used as the growth medium. Many species, such as *S. salivarius* and *S. mutans*, reside in oral and nasal cavities, and contribute to tooth decay (dental caries). To distinguish the nonpathogenic streptococci of fecal origin, these species are now classified as *Enterococcus.*

Streptococci classically have been classified into about 11 **Lancefield Groups**, named for Rebbeca Lancefield who classified the streptococci based upon antigenic properties of their cell walls. Species within each group share some other properties as well. For example, groups A, B, C and G are typically β-hemolytic, and many are pathogenic. Other groups show α- or no hemolysis. Along with their antigenic properties, streptococci in some Lancefield groups share metabolic properties that can be used in their identification.

Metabolic characteristics of four Lancefield groups

Group	Hemolysis	PYR metabolism	Esculin metabolism	Bacitracin sensitive
A	β	+	–	+
B	β	–	–	–
C	β	–	–	–
D*	α or γ	+	+	–

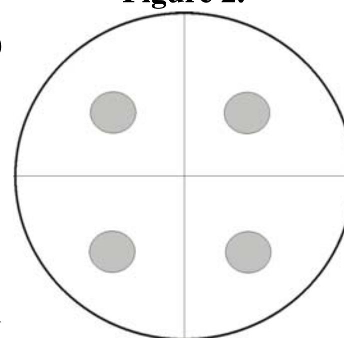
* D group also includes *Enterococci*, which are no longer classified as *Streptococci*.

1. Determination of hemolytic propeties

Supplies

Streptococcus pyogenes (green cap)
Alcalegenes faecalis (clear cap)
Enterococcus faecalis (purple cap)
Your semester unknown
1 Blood Agar plate

Figure 2.



Procedure

1. Quadrasect the plate of Blood Agar, in the manner shown in

Figure 1.

2. Label sectors for *S. pyogenes*, *A. faecalis*, *E. faecalis,* and your semester unknown.
3. Inoculate a ~2 cm diameter area (relatively large) in the center of each sector with the appropriate bacterium.
4. Incubate at 37°C for 24 - 48 hours.
5. Record your observations in Table 1. This plate will be used below for Parts 3 and 4.

2. Immunological differentiation of Streptococci

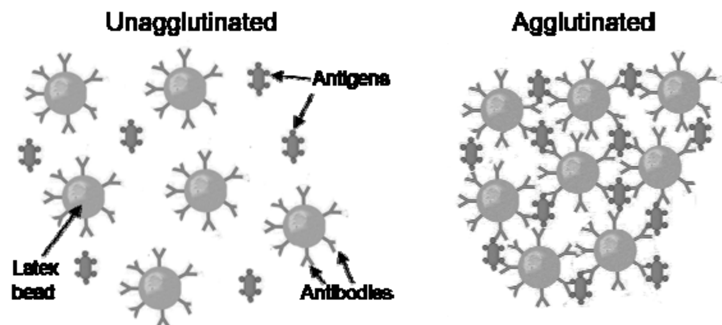
One modern method for rapidly identifying pathogens looks at the types of surface molecules, called **antigens**, on the cells. As described above, different Lancefield group streptococci have different types of carbohydrates on their surface, and these can be distinguished using antibodies that react only with the specific types of antigens. In this activity you will use an immunological test to identify the Lancefield grouping of several test organisms.

To perform the test, several reagents are first used to expose the antigen on the surface of the cells. Antibodies coupled to microscopic-sized latex beads are then added. If the antibodies can react with the antigen, the antibodies (and attached beads) become cross-linked together (called **agglutination**). The agglutination can be visibly detected.

Supplies

Streptococcus pyogenes(plate)
Enterococcus faecalis (plate)
Semester unknown (if gram-+ coccus)
or *Alcaligens faecalis*
3 13mm test tubes
3 plastic inoculating loops
2 agglutination test cards
16 wooden tooth picks
Agglutination test reagents

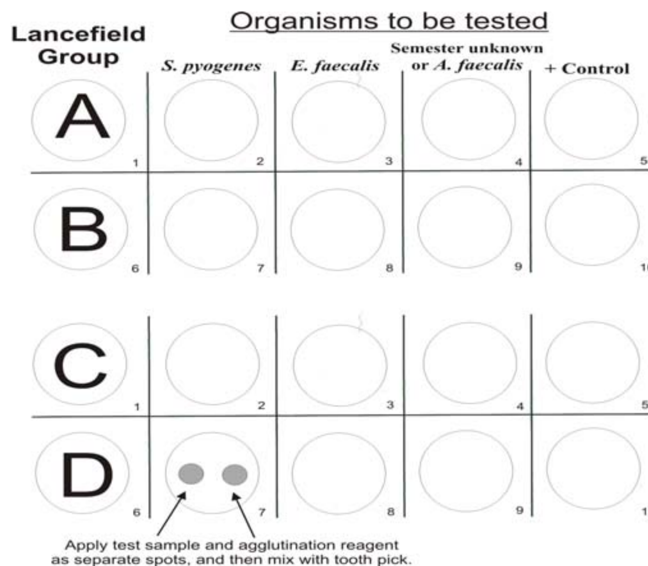
Figure 3. Agglutinated and unagglutinated latex beads.



Procedure – this will be performed when you return to the lab. You will test each species for the presence of Lancefield group antigens A - D.

1. Label three 13mm test tubes, one for each species to be tested. **Use your semester unknown if it is a Gram-+ coccus in place of *A. faecalis*.** Label the 2 test cards as shown in Figure 4.

Figure 4. You will use 2 agglutination test cards, labeled as shown here.



To expose the bacterial antigens, <u>for each species to be tested</u>:

2. Add one drop of Extraction Reagent 1 to a 13 mm test tube – be sure that the drop goes to the bottom of the tube.
3. Using a plastic inoculating loop, transfer a loopful of bacterial growth into the extraction reagent, and twirl the loop to mix. There should be enough bacteria for the reagent to become turbid.
4. Add one drop of Extraction Reagent 2 to the tube, and mix by tapping the tube with a finger for 10 seconds.
5. Add five drops of Extraction Reagent 3 to each tube, and mix as in step 4.

Adding agglutination reagents and bacterial samples to the cards:

6. Resuspend the Latex Agglutination Reagents A, B, C, and D by inverting the tubes. Dispense one drop of each reagent onto circles (**off center – see Fig 4**) of the appropriate row on the test cards. **Each row will receive either reagent A, B, C or D in all four circles.**
7. Using a plastic squeeze pipette, for each column place one drop of each bacterial suspension next to the drop of latex suspension in each circle. Add one drop of the positive control reagent to the appropriate column also.
8. Mix the agglutination reagent and the bacterial sample with a wooden tooth pick over the complete area of the circle. A different tooth pick should be used for each circle. Dispose of the tooth picks in your disposal jar.
9. Gently hand rock the cards, allowing the mixture to flow slowly within the ring area.

Results

After several minutes, look for clumping of the blue latex particles; this should be obvious in each of the positive control circles. A reaction with only one of the latex reagents indicates an identification of the Lancefield group. Record your results in Table 1.

3. PYR and Esculin metabolism

Some Lancefield groups can be distinguished by their metabolic capabilities. For example, esculin metabolism and PYR; esculin is an organic molecule which can be metabolized by some bacteria to release sulfur compounds, whereas PYR (L-pyrrolidonyl-β-naphthylamide) is a colorimetric test for the enzyme pyrrolidonyl peptidase. Using a single rapid test, these properties can be identified in about 30 minutes.

Supplies

Streptococcus pyogenes, *Enterococcus faecalis* and semester unknown
from plate prepared in Part 2
3 PYR/esculin screen tubes
PYR reagent (on side bench)

Procedure: this test will be performed when you return to the lab

1. With an innoculating loop, pick up a loop of bacterial growth and macerate it into the medium along one edge of the vial.
2. Incubate in the 35°C water bath until the end of the lab period.
3. Observe for the formation of a brown/black color within the medium. This is indicates a positive result for esculin metabolism.
4. Add two drops of the PYR reagent to the microtube; a positive PYR test is indicated by formation of a bright cherry-red color in the reagent within 5 - 10 minutes.
5. Record your results in Table1.

III. Characteristics of *Staphylococcus*

The spread of methicillin-resistant *Staphylococcus aureus* (MRSA) has greatly elevated concern over this organism in the last decade. MRSA are generally resistant to all antibiotics except vancomycin; but some new strains that have emerged are resistant to this antibiotic as well. Staphylococci are nonmotile Gram-positive cocci, arranged in irregular clusters. The natural habitat for many species is the skin of warm-blooded animals. Certain strains of *Staphylococcus* can cause serious human diseases, including human mastitis (infection of lactating breasts), boils, impetigo and other skin infections, and toxic shock syndrome. The pathogenic properties of staphylococci generally are attributable to the production of potent exotoxins, such as hemolysin and coagulase (which causes clotting of blood). This organism is also an important cause of heat stable food poisoning.

4. Isolating *Staphylococcus* from the Skin

Mannitol-salt agar is used to isolate pathogenic strains of *Staphylococcus*. *Staphylococcus* can grow in the presence of 7.5% sodium chloride incorporated in the medium. Certain pathogenic strains of *Staphylococcus* can ferment mannitol with the production of acid, and this characteristic is distinguished by the formation of a yellow halo around the bacterial colony. Most nonpathogenic species do not produce a yellow zone. This medium would therefore be selective and differential. Why?

Mannitol Salt Agar	
Beef-extract	1 g/L
Proteose peptone #3 . .	10
NaCl	75
Mannitol	10
Phenol-red	0.025
Agar	15

Supplies

2 sterile cotton swabs
2 Mannitol Salt Agar plates   tube of sterile H_2O (black cap)

Procedure

Each team member should innoculate a Mannitol Salt Agar plate from their skin.

1. Lightly dampen a cotton swab, use it to wipe both nostril grooves.
2. Confluently swab the surface of the Mannitol Salt Agar plate using the clinical streaking method.
3. Incubate the plates at 37°C for 24 - 48 hours.
4. Perform a Catalase test (described below) on one of the isolated colonies.

5. Plasma Coagulase

Coagulase is an enzyme that causes the proteins of blood serum to coagulate around a pathogen, probably as molecular camouflage from immune detection. In the coagulase test a sample of the bacteria is transferred to a tube containing blood plasma. "Gelling" of the plasma will occur if coagulase is present.

Supplies

2 tubes of coagulase plasma (pink cap)   your semester unknown
Staphylococcus aureus (on plate)

Procedure

1. Label 2 tubes of coagulase plasma as *Staphylococcus aureus* and your semester unknown.
2. Using your inoculating loop, transfer a loopful of bacteria from the appropriate cultures to the tubes.
3. Incubate plasma at 37°C for 24 hr. Check for coagulation by gently rocking the tube.

6. Catalase test

Catalase is an enzyme produced by most aerobic organisms to detoxify H_2O_2 that accumulates in the cell. When cells containing catalase are mixed with H_2O_2, the peroxide is converted to H_2O and O_2, with vigorous bubbling. (The same reaction occurs when H_2O_2 is applied to a cut or wound.) The catalase test is relatively simple and useful for distinguishing *Staphyloccccus* from *Streptococcus* and *Enterococcus*.

Supplies

clean microscope slide
dropper bottle of H_2O_2
Your semester unknown
Plate cultures of *Enterococcus faecalis* and *Staphylococcus aureus*

Procedure

1. Place a drop of H_2O_2 on a microscope slide and stir in a loopful of cells to be tested.
2. Vigorous bubbling is a positive result. Little or no bubbling is negative.
3. Discard the microscope slide in your disposal jar.

IV. Gram-staining *Neisseria* and other cocci

Neisseria are among the few Gram-negative cocci (although it has a slight tendency to resist decoloration; there may be a scattering of Gram-positive-appearing cells). Most species occur as pairs of slightly flattened dipplococci. Human mucosyl membranes, such as in the nose and urinary tract are common habitats, and *N. meningitidis* and *N. gonorrhoeae* are important human pathogens. *N. subflava* occurs indigenously in humans, and is occasionally an opportunistic pathogen.

7. Gram stains

It takes practice to learn to reliably distinguish coccus-shaped bacteria. Gram stain the following bacteria to study and carefully compare the shape and arrangements of the coccus-shaped bacteria, *Enterococcus*, *Staphylococcus*, and *Neisseria*. This is an excellent time to re-Gram stain your semester unknown! Record your results.

Organisms to Gram stain (as good practice, you may wish to Gram stain both broth and plate cultures if available)

Enterococcus faecalis
Staphylococcus aureus
Neisseria subflava
Your semester unknown

Names: ___________________________________

Results for Indigenous Bacteria and Pathogens Lab

✶✶Indicate where your results do not agree with your expectations, and write a brief explanation of the discrepancy✶✶

Survey of hemolytic bacteria

What was the most common type of hemolysis observed?

Were β-hemolytic colonies observed? If so approximately how many?

Do a Gram stain on one of the colonies (a β-hemolytic colony if present) and record the Gram reaction, shape and arrangement of the cells:

Table 1. Hemolysis and Lancefield group analysis.

Test organism	Hemolysis (α, β or γ)	Esculin (+ / –)	PYR* (+ / –)	Agglutination Lancefield Group
Streptococcus pyogenes	_____	_____	_____	_____
Appearance of hemolytic zone:				
Enterococcus faecalis	_____	_____	_____	_____
Appearance of hemolytic zone:				
Alcalegenes faecalis	_____	_NA_	_NA_	_____
Appearance of hemolytic zone:				
Semester unknown	_____	_____	_____	_____**
Appearance of hemolytic zone:				

* Do not mistake orange color of the reagent for the 'cherry-red' color of a positive result.
** Perform only if your unknown is a gram positive coccus, otherwise indicate NA.

4. Isolation of *Staphylococcus* from the skin

Appearance of the most common colony type (use proper terms for colony morphology)

Was mannitol fermentation observed for any colonies?

Result of catalase test:
Was this as expected for *Staphylococcus*?

5. Coagulase test

Test organism	Appearance of serum	Coagulase (+ or –)
S. aureus	________________________	_______
Semester unknown	________________________	_______

6. Catalase test

Test organism	Catalase (+ or –)
E. faecalis	______
S. aureus	______
Semester unknown	______

7. Grams Stains

Draw quality, meaningful diagrams showing the typical appearance of these cells and their arrangements; state results of Gram stain (reaction shape, arrangement); identify some subtle features that distinguish the appearance of each group of bacteria.

E. faecalis

S. aureus

Neisseria subflava

Your semester unknown

Also turn in answers to these questions (typed):

1. Considering the appearance of the mouth culture survey plate that you prepared in Part I and the discussion in the unit Introduction, what do perceive to be some of the challenges to the isolation and identification of potential pathogens from bodily fluids?

2. Suppose that you performed an agglutination test on a β-hemolytic colony from your mouth survey plate; and no agglutination occurred for any Lancefield antigen. Give two reasonable explanations for this result.