Laboratory Exercise # 11:
Differentiation of the Species Staphylococcus and Streptococcus

Purpose:

The purpose of this laboratory exercise is to explore the differences between Staphylococcal species and Streptococcal species.

Introduction:

In this exercise we continue our use of specialized media and use some biochemical testing to differentiate the two genera Staphylococcus and Streptococcus.

Blood agar is an example of an enriched, differential media. It contains Trypticase Soy agar with 5 or 10% Sheep blood added to the agar. When bacteria are grown on this enriched media, their appearances may differ, due to their ability to utilize the red blood cells (RBC's) for their metabolic purposes. When bacteria are able to utilize RBC's there is a change in the appearance of the agar surrounding the individual colonies. This change is called hemolysis, meaning the break down (lysis) of the hemoglobin within the RBC's. The types of hemolysis include:

- **Beta hemolysis**: the complete destruction of the hemoglobin within the RBC's yielding a clearing around the individual colonies. **Media may look yellow: that is the color of the Trypticase agar.**

- **Alpha hemolysis**: partial destruction of the hemoglobin within the RBC's yielding a greenish, brown coloration around the individual colonies.

- **Gamma hemolysis**: no break down of the hemoglobin within the RBC's yielding red coloration around the individual colonies.

Both Staphylococcus and Streptococcus species have the ability to break down hemoglobin to differing extents; therefore, other methods of differentiation of these species are required. These methods can be the use of other selective/differential media or the use of biochemical tests. Both of these methods will be presented in this laboratory exercise.
**Mannitol Salt Agar (MSA)** is a selective/differential media that inhibits the growth of gram negative bacteria. Only a few bacteria can tolerate the 7.5% NaCl found within this media. The high salt content selects for *Staphylococcus* and a few salt tolerant enterococci. The presence of the sugar mannitol gives this media its differential properties. When mannitol is fermented (utilized by the bacteria) microorganisms release acidic byproducts that change the pH of the surrounding media. The pH change is indicated by a change in the pH indicator (phenol red) from pink to yellow.

Biochemical testing is different from the use of differential and selective media. Unlike selective/differential media, which can be used on a mixed culture, biochemical testing can only be done on pure cultures. The inadvertent use of a mixed culture in biochemical testing will lead to erroneous results.

The simplest biochemical test that is used to differentiate between *Streptococcus* and *Staphylococcus* species is the **Catalase test**. In this test we are testing for the ability of the bacteria to produce the enzyme catalase. This enzyme breaks down hydrogen peroxide into water and oxygen. Hydrogen peroxide, as well as superoxides, can be metabolic end products of the production of ATP by the electron transport chain. If oxygen only receives one electron it can become a superoxide instead of water. Superoxides are toxic to the cell, but are normally converted to hydrogen peroxide by the enzyme **superoxide dismutase**. This enzyme is present in all aerobic organisms, but this still leaves the toxic end product hydrogen peroxide. If the microorganism has the ability to produce catalase, then the hydrogen peroxide is converted to other harmless end products of water and oxygen. The chemical equation for this reaction is:

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

A positive reaction is observed by watching for the production of bubbles. *Staphylococcus* species produce catalase, but *Streptococcus* species do not.

Now we have been able to separate the two genera biochemically. Next we will identify different species within each genus. The *Staphylococcus epidermidis* is a common component of our skin flora, but there is also another member of the genus that can be highly pathogenic, *Staphylococcus aureus*. To differentiate these two species we use the **Coagulase test**. Coagulase is an enzyme that causes fibrin in plasma to clot. *S.aureus* is the only *Staphylococcus* species that produces coagulase.
Streptococcus species can be either pathogenic or normal flora and can be differentiated biochemically by several different tests. We will be performing the following tests:

**Bacitracin sensitivity:** used to identify Group A strep, the cause of strept throat. Group A strep are the only species group that are sensitive to this antibiotic and can be detected by a zone of inhibition around the Taxo A disc.

**Bile esculin testing:** incorporates two tests; the ability to grow in 40% bile and the ability to hydrolyze esculin to esculatin that then combines with Fe^{3+} to form a black precipitate. This media is used to identify Enterococcus which can cause urinary tract as well as opportunist infections. A black precipitate is considered a positive test.

**6.5% NaCl testing:** used to speciate Enterococcus. The high salt content is tolerated by Group D enterococcus, but not by non-Group D enterococcus. The media changes from purple to yellow when growth occurs.

**Optochin sensitivity:** Streptococcus pneumoniae is the only strept species that is sensitive to the drug optochin (ethylhydrocupreine hydrochloride). This can be detected by a zone of inhibition around a Taxo P disc.

**Materials:**

1 Sheep blood agar plate streaked with the following for hemolysis demonstration:

- Staphylococcus aureus
- Staphylococcus epidermidis
- Streptococcus bovis (plus three for class use)
- Enterococcus faecalis
- Streptococcus pyogenes with Taxo "A" disk
- Streptococcus pneumoniae with Taxo "P" disk

3 TSA plates streaked with the following:

- Staphylococcus aureus
- Escherichia coli
- Staphylococcus epidermidis
- Enterococcus faecalis

6.5% salt - 2 per pair of students
Bile Esculin slants - 2 per pair of students
3% Hydrogen peroxide
Rabbit plasma - 2 per pair of students
Mannitol Salt agar - 3 per pair of students
**Procedure:** To be done in pairs

Day # 1:

1. Run a Catalase testing on one of the Staphylococcal species and one of the Streptococcal species, using the following method.
   a. Using a sterile loop remove a small amount of the microorganism from the agar plate provided onto a clean glass slide.
   b. Place a drop of hydrogen peroxide onto the bacteria and observe for the production of immediate bubbles.
   c. Record your observations on the data sheet.

2. Streak the following microorganisms on Mannitol salt agar using a four quadrant streak plate method:
   - Staphylococcus aureus
   - Staphylococcus epidermidis
   - Escherichia coli

   Incubate the plates at 37°C until the next lab period

3. Run a coagulase test on both the Staphylococcus aureus and the Staphylococcus epidermidis cultures, using the following method.

   Inoculate two (2) plasma tubes one with S.aureus and the other with S.epidermidis. Incubate at 37°C until the next laboratory period.

4. Using the cultures of Enterococcus faecalis and Streptococcus bovis inoculate two slants of bile esculin and two tubes of 6.5% NaCl. Incubate the tubes at 37°C until the next lab period.
Day # 2:

1. Observe the following microorganisms on a Sheep blood agar plate and record the hemolysis patterns on the data sheet:

   Staphylococcus aureus
   
   Staphylococcus epidermidis
   
   Enterococcus faecalis
   
   Streptococcus bovis

2. Observe the two cultures of Streptococcus pyogenes and Streptococcus pneumoniae. Record your observations for hemolysis reactions and disc sensitivities on the data sheet.

3. Observe and record the results of the Mannitol Salt Agar plates on the data sheet.

4. Observe the results of the coagulase tests and record the results on the data sheet.

5. Observe the results of the Bile esculin and 6.5% Salt tests and record the results on the data sheet.

Data: For Staphylococcus Identification

<table>
<thead>
<tr>
<th></th>
<th>Hemolysis</th>
<th>Mannitol Salt Agar</th>
<th>Catalase</th>
<th>Coagulase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Growth</td>
<td>Sugar Use</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*** Mannitol Salt Agar for Escherichia coli: ______________________________
Data: For *Streptococcus* Identification:

<table>
<thead>
<tr>
<th></th>
<th>Hemolysis</th>
<th>Catalase</th>
<th>Taxo A</th>
<th>Taxo P</th>
<th>Bile Esculin</th>
<th>6.5% NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterococcus faecalis</strong></td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus bovis</strong></td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus pyogenes</strong></td>
<td></td>
<td></td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td>N/A</td>
<td></td>
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</tr>
</tbody>
</table>

*** Students are required to record all results even if they didn't do that particular microorganism!!

Questions:

1. Explain how you determine whether a culture is positive for *Streptococcus pyogenes*.

2. If you wanted to differentiate *Enterococcus faecalis* and *Streptococcus bovis* which test would you use?

3. Write the equation that represents the reaction that takes place when you test for the presence of the enzyme catalase.

4. Define the following:

   Beta hemolysis -

   Alpha hemolysis -

   Gamma hemolysis -

5. Why is Mannitol Salt Agar considered a selective differential media?

6. Explain what a positive coagulase test looks like.
Staph/Strep Review

Hemolysis

- +
- Neg.

Used to differentiate: ___________________________

Catalase

Rx:

+ +
Neg. =

Used to differentiate: ___________________________

Coagulase

+ +
Neg. =

Used to differentiate: ___________________________
Mannitol Salt Agar

Selective = _____________________
Differential = ___________________
Used to differentiate ________________

Taxo A
Antibiotic
Sensitivity + Beta Hemolysis = ________________

Taxo P
Antibiotic
Sensitivity + Alpha Hemolysis = ________________

Bile Esculin

+ = ________________
Neg. = ________________
Used to differentiate ________________

6.5% NaCl

+ = ________________
Neg. = ________________
Used to differentiate ________________

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