LABORATORY: Differentiation of Streptococcus
Skills= 17.5 + 7= 24.5 POINTS

Objectives:

At the completion of this activity, the student should be able to:

1. Observe and describe the Gram stain morphology for each *Streptococcus* species studied.
2. Explain the significance of using sheep blood agar in the characterization of the streptococci.
3. Describe the hemolytic properties used to characterize the streptococci.
4. Identify the following streptococci with the type of hemolysis they produce:
    a. Group A Streptococcus (*S.pyogenes)*
    b. Group B Streptococcus (*S.agalactiae)*
    c. Group D Streptococcus, *Enterococcus* (*E.faecalis)*
    d. Group D Streptococcus, non-*Enterococcus (S.gallolyticus/S.bovis*)
    e. Viridans streptococci (*S.mitis*, *S.thermophilus, S. anginosus, S. sanguinis*)
    f. *Streptococcus pneumonia*
5. List the streptococci that give positive reactions for each of the following biochemical tests:
    a. Catalase
    b. Bacitracin sensitivity
    c. Bile-esculin test
    d. 6.5% NaCl tolerance
    e. PYR test
    f. Optochin sensitivity
6. State the principle of the rapid Strep test.
7. Analyze a throat swab specimen for the presence of *S. pyogenes.*

Materials:

Blood agar cultures of:

a. *S. viridans* (*S.mitis*, *S.thermophilus, S. anginosus, S. sanguinis*)
b. *S. pyogenes* (Group A)
c. *S. pneumoniae*
d. Group D Enterococcus (*E. faecalis*)
e. Group D Nonenterococcus (*S. gallolyticus/ S. bovis*)
f. *S. agalactiae* (Group B)

Gram stain materials
6 glass microscope slides
Microscope
Immersion Oil

1 Vial of DiH_20
3% hydrogen peroxide (catalase)
1 Bacitracin disk (Taxo A)
2 blood agar plates (BAP)
2 PYR tests
3 tubes Bile Esculin Azide
2 tubes 6.5% NaCl
2 Optochin disk (Taxo P)
Prolex Strep Typing Kit
Rapid Strep Kit
1 Unknown Patient Throat Swab (submitted in a dual swab collection system)

References:

1. Mahon and Manuselis, Textbook of Diagnostic Microbiology, Third Edition, Chapter 15
2. Clinical Microbiology Procedures Handbook, volumes 1 and 2, ASM Press, 1992.
3. Remel PYR kit product insert
4. Prolex Strep Grouping product insert
5. BD BBL Taxo Discs for Differentiation of Group A Streptococci product insert

Discussion:

Organisms of the genus Streptococcus are capable of inducing infection in every organ and tissue of the body. Most pathogenic species of the streptococci are aerobic, but a few are anaerobic or microaerophilic. Streptococci can be divided into groups based on hemolytic activity, biochemical tests, and colony morphology.

Procedure:

1. Colony Morphology, Hemolytic Properties, Gram Stain, and Catalase

In addition to being grouped according to serological properties, Streptococci may also be classified according to the type of hemolysis produced on sheep blood agar. This type of grouping is referred to as Brown's classification. The four types of hemolysis are alpha, beta, gamma, and alpha prime.

Alpha hemolysis is caused by partial lysis of the red cells in blood agar, allowing hemoglobin to be released and its subsequent degradation to biliverdin. It is characterized by an indistinct zone around the colony with a **greenish** or **brownish** discoloration.

Beta hemolysis is caused by complete lysis of the red cells characterized by a clear, colorless zone around the colony.

Gamma hemolysis is actually a misnomer in that there is no apparent hemolysis or discoloration around the colony of blood agar.

a. Working in pairs, describe the colony morphology and hemolytic properties of each Streptococcus isolate in the report form.
b. Prepare smears of each isolate. You will make a total of six (6) smears, putting two (2) smears on each slide.
c. Gram stain each isolate and enter the results in the report form.
d. Perform a catalase test on each strep species. One (1) slide can be used for two (2) tests. *Be careful not to take any blood agar with the colonies!* This can result in a false positive.

2. Group D Streptococcus Differentiation

Group D streptococcus is a classification of streptococci that may produce alpha, beta, or gamma hemolysis on sheep blood agar. It may be isolated from blood in subacute bacterial endocarditis, from specimens in urinary tract infections, or from wounds and deep abscesses.

The Group D streptococci are divided into two groups: (1) Enterococci and (2) Nonenterococci. It is important that this differentiation be made because enterococci are less susceptible to penicillin than are other streptococci. In some cases of severe infection, combined therapy with two or more antimicrobial agents must be used.

Bile Esculin Test

The bile-esculin test is based on the ability of certain bacteria, notably the Group D streptococci, to hydrolyze (break down) esculin in the presence of 1% to 4% bile. Bacteria capable of growing in bile and also hydrolyzing esculetin produce glucose and the aglycone esculetin in an appropriate medium. Esculetin reacts with an iron salt to form a dark brown or black complex.

a. Streak three (3) bile esculin slants heavily with *S.agalactiae, E.faecalis,* and Grp. D *Non-enterococcus*. Leave the **caps loosened** to ensure adequate aeration.
b. Incubate at 35^0 C for 24 hours.

Interpretation: Observe the slant for diffuse blackening of the slant, which is a positive reaction. Record results as either “positive” or “negative” in the report form.

6.5% NaCl Tolerance

Once a streptococcus is identified as belonging to the Group D (positive bile esculin reaction) streptococci, it is necessary to determine whether it is an enterococcus or one of the non-enterococcus species. Enterococci will grow and produce acid in heart-infusion broth containing 6.5% sodium chloride; non-enterococci are inhibited from growing in this concentration of salt.

a. Inoculate two tubes of 6.5% NaCl medium, using the loop. One tube will be inoculated with *E.faecalis* and one tube, Grp. D *Non-enterococcus.* Leave the **caps loosened** to ensure adequate aeration.
b. Incubate at 35^0 C for 48 hours.

Interpretation: Observe for turbidity in the medium which indicates a positive test. Record results as either "positive" or "negative" in the report form.

With the bile-esculin and salt tolerance tests, three presumptive identifications are possible:

Presumptive ID	Bile esculin	6.5 % NaCl
Presumptive *S.viridans*	=	=
GDNE **Group D Non-Enterococcus**	+	=
GDE **Group D Enterococcus**	+	+

PYR Tests

The PYR test is a qualitative procedure to determine the ability of streptococci to enzymatically hydrolyze L-pyrrolidonyl-β-naphthylamide by the enzyme pyrrolidonyl peptidase. PYR tests are often used as an alternative to bile esculin and 6.5% NaCl tolerance as it provides a rapid, presumptive, result.

PYR Disc

a. Place 1 disc onto a slide and moisten slightly with diH_20. Do not over-saturate the disc.
b. With an applicator stick or sterile inoculating loop remove a heavy paste of Enterococcus from a fresh culture.
c. Wait 2 minutes at room temperature, then add 1 drop of color developer reagent.
d. Wait 2 minutes for color to develop.

Interpretation: The appearance of a dark pink to red color within 2 minutes is a positive reaction. No color change indicates a negative result. Results should be reported as either "positive" or "negative" in the report form.

e. Repeat steps a-d using the Viridans streptococci.

3. Optochin (Taxo P) Susceptibility

Optochin (ethylhydroxycupreine hydrochloride), a quinine derivative, selectively inhibits the growth of *S. pneumoniae* in very low concentration. Optochin may inhibit other alpha-hemolytic streptococci, but only at higher concentrations.

a. Split a BAP and label the plate with *S.pneumonia* on one side, and *Streptococcus* viridians on the other.
b. On one half of the plate, inoculate *S.pneumoniae* heavily.
c. On the other half of the plate, inoculate *Strep viridans* heavily.
d. On one half on the BAP, place a disk impregnated with optochin (Taxo P) on the thickly inoculated area of *S. pneumoniae*.
e. On the other side of the BAP, place a disk impregnated with optochin (Taxo P) on the thickly inoculated area of *S. viridians.*
f. Incubate overnight in a 35^{O}C, CO_2 incubator.

Interpretation: A zone of inhibition of growth ≥ 14 mm is considered a positive test for *S. pneumoniae*. Report results as positive in the report form.
No zone of inhibition is consistent with alpha streptococci other than *S. pneumoniae*. Report results as either negative in the report form.

4. Streptococcus Typing Kits

B- hemolytic streptococci can be differentiated into Lancefield groups based on specific carbohydrate antigens. This procedure uses an acid latex to extract the antigens for a rapid ID. The latex test particle will agglutinate in the presence of a homologous antigen.

a. Resuspend the test latex reagents by gently inverting the dropper bottle several times.
b. Label one test tube for each isolate to be tested.
c. Add one (1) drop of Extraction Reagent 1 to each tube.
d. Select 1-4 isolated beta-hemolytic colonies using a disposable loop or needle and suspend them in Extraction Reagent 1.
e. Add one (1) drop of Extraction Reagent 2 to each tube.
f. Mix the reaction by gently swirling the tube for 5-10 seconds.
g. Add five (5) drops of Extraction Reagent 3 to each tube and mix by swirling the tube for 5-10 seconds.
h. Dispense one drop of each group latex reagent onto separate circles on the the card, labelling each isolate to be tested.
i. Using a pipette, place one drop of extract *BESIDE* each drop of latex reagent.

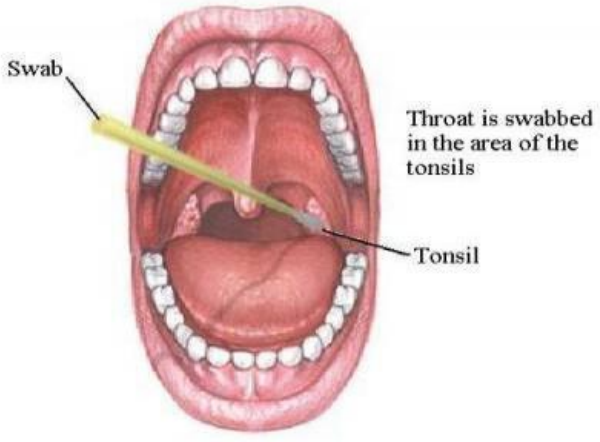
j. Mix the latex and the extract with the sticks provided, using the complete area of the circle. A new stick should be used with each test circle.
k. Gently rock the card allowing the mixture to flow slowly over the entire testing area.
l. Observe for agglutination for up to one minute.

Interpretation: Rapid strong agglutination of the blue latex particles within one minute indicates a positive result for that specific streptococcal isolate. Report as "Group ___positive" in the report form. A weak reaction should be repeated with a heavier inoculum. No agglutination of the latex particles is a negative result. Repot as negative in the report form.

5. Rapid Strep Tests

Throat swab specimens **must** be collected properly in order to obtain accurate results. **Do not use calcium alginate swabs**. For best results use a dacron or rayon swab for **both** the patient specimen and the **Positive Control**. Cotton swabs are not recommended as they tend to release fibers making the agglutination difficult to interpret; in addition, some are too tightly wound to deliver adequate volumes of extract onto the test plate.

The swabbing technique used to collect the specimen is as important to the test result as is the performance of the test. The throat swab specimen must be collected under direct visualization with good lighting. The pharynx and tonsils must be adequately exposed and vigorously rubbed with a sterile rayon or Dacron® tipped swab. The tongue, uvula, and cheeks must be avoided. Any exudate present should also be touched with the swab. The swab can be tested immediately or held up to 72 hours.



a. Note the kit brand and expiration date in your report sheet. Follow the kit instructions provided by the instructor.
b. The instructor will perform the external quality control. Log the QC results in the report form.
c. Note the patient name and ID on the report form.
d. Using **only ONE** of the patient swabs from the collection system, perform the test.

Interpretation: A positive result will be reported as "Grp. A Recovered." Record results on the report form. If the patient sample is negative, report as "Negative for Grp. A Strep."

e. If the patient sample is negative, a reflex to a throat culture with bacitracin (Taxo A), will be performed using the second swab. Group A Streptococcus may be differentiated from other Streptococci by its sensitivity to low concentrations of bacitracin. A paper disk containing 0.04 U bacitracin (Taxo A) is used for this test.
e. Inoculate a BAP in the first quadrant using the patient swab, then streak for isolation.
g. Gently press a "A" disk to the second inoculated quadrant of the BAP using forceps.
f. Incubate the plate overnight in a CO_2 incubator at 37 ^{O}C.

Interpretation: Any zone of inhibition of growth around the disk is positive identification of Group A Streptococcus (*S.pyogenes*). This means that the organism is susceptible to the bacitracin. Indicate the results for this organism as "positive" in the report form. Absence of a zone of inhibition means that the organism is not susceptible or killed by the bacitracin. Report this out as "negative" in the report form.

Presumptive Identification of *Streptococci*

Test	Group A	Group B	Enterococcus	Grp. D, not Enterococcus	Viridans, Non-Grp. D	Pneumococci
Hemolysis	β	β *	α,β,γ	α,γ	α,γ	α
Bacitracin	+	- *	- *	- *	+ *	±
Bile-Esculin	-	- *	+ *	+ *	- *	-
6.5% NaCl	-	- *	+ *	- *	- *	-
Optochin	-	-*	- *	- *	- *	+
Catalase	-	- *	- *	- *	- *	-
PYR	+	- *	+ *	- *	- *	-

*** Occasional exceptions occur.**

Quality Control

Quality control ensures that the information generated by the laboratory is accurate, reliable, and reproducible. This is accomplished by assessing the quality of the specimens, monitoring the performance of test procedures, reagents, media, and personnel. For the reagents used in this lab, QC should be performed weekly and on each new lot or shipment of the product using ATCC control organisms. Results should be reported in the QC log. Components of kit tests should not be interchanged with those from another kit.

Technical Notes

Most clinical laboratories do not use all of these methods to identify members of the genus Streptococcus. In the consideration of time, rapid tests, such as the strep typing or PYR are used instead of tube testing which requires a 24-hour incubation time.

Report Form: *Streptococcus* Differentiation
Points= 17.5

Name _____ Date _____
Note: Greyed out tests are not performed.

Test	**Group A: *S. pyogenes***	**Group B: S. agalactiae**	**Enterococcus**	**Grp. D, Non-Enterococcus**	**Viridans, Non-Grp. D**	**Pneumococci**
Hemolysis						
Colony Morphology						
Gram Stain						
Catalase						
Bile Esculin						
6.5% NaCl						
PYR						
Optochin (Taxo P)						
Strep Typing						

Grading Rubric:
- Each test result is worth 0.5 points

Lab: Streptococcus- Rapid Strep Testing
Report Form
Points= 7

Name _____ Date _____

Kit brand:_____

Expiration date:_____

	Results
Positive Control	
Negative Control	
Patient name:	
Patient ID:	
Patient Rapid Strep result:	
Bacitracin result, if indicated	

Grading Rubric:
Each item listed is worth = 1 point:

- Kit brand
- Kit Expiration Date
- Pos & Negative Control
- Patient Name & ID
- Rapid Strep results

Name:_____
Date:_____

Lab: Differentiation of Streptococcus
Points= 10
Study Questions

1. What biochemical test differentiates *Streptococci* from *Staphylococci?* (1 pt)

2. Describe what α, β, and gamma hemolysis look like on SBA (sheep blood agar). Why is this reaction important in the ID of streptococci? (4 pts.)

3. Based on the below descriptions, identify the organism. (3 pts.)

Gram stain reaction	Media	Biochemicals	Organism
GPC	BAP: beta hemolytic	**Catalase:** neg **PYR**: pos	
GPC	BAP: gamma hemolytic	**Catalase**: neg **PYR**: neg **Bile esculin**: turned black **6.5% NaCl**: no growth	
GPC	BAP: gamma hemolytic	**Catalase**: neg **PYR**: pos **Bile esculin**: turned black **6.5% NaCl**: growth	

4. Taxo A is _____. (1 pt.)

5. Taxo P is _____. (1 pt.)