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TITLE: Basics of Differentiation of Gram-positive Cocci

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Slide 1:

Hello, my name is Dr. Karen Krisher. I am an associate professor in the Clinical Laboratory Science program at Wayne State University. Welcome to this Pearl of Laboratory Medicine on "Basics of Differentiation of Gram-positive Cocci."

Slide 2: Gram-positive Cocci

In this presentation, we will discuss the fundamentals of the primary identification of those microorganisms that are members of four main families of Gram-positive cocci, the *Micrococcaceae*, the *Staphylococcaceae*, the *Streptococcaceae,* and the *Enterococcaceae*. The two unifying characteristics of these four families are their coccoid shape and the fact that they stain purple in the Gram's method for visualizing bacteria microscopically. These organisms range in virulence potential from commensal organisms found on the skin and mucous membranes to pathogens associated with serious infections. The previous classification of these organisms was much simpler; however, with the use of more advanced genetic sequencing methods, these gram-positive cocci have expanded into 4 separate families and their associated genera. Although many of the members of these families have low pathogenicity and are classified as causes of opportunistic infections, those organisms most often cited as pathogens are listed on this slide. This presentation will focus on the laboratory tests useful for the differentiation among the families as opposed to the more complicated differentiation and identification of the organisms within the different genera.

Slide 3: Differentiation of Gram-positive Cocci

The differentiation of the Gram-positive cocci encompasses 4 main exercises used to compare and contrast the characteristics of each family:

1. Colony morphology, the elucidation of the characteristics of the growth of the bacterium on agar medium, in most cases trypticase soy agar containing 5% sheep blood (sBAP). Growth can produce colonies of a certain size and consistency, as well as pigmentation.
2. Included in the observation of the morphology of the colony is the effect that the bacterial growth has on the sheep erythrocytes in the agar medium. We will discuss this shortly.
3. The microscopic morphology of the organisms as visualized using the Gram's stain can give initial clues as to the possible identification of the organism.

4. The categorization of the Gram-positive cocci based on their possession of the enzyme catalase, a quality which can be easily assayed in the lab.

These four tests will, as we shall see, allow for the differentiation of the families of organisms and will guide the laboratorian to other more definitive tests for the identification of the organism.

Slide 4: Differentiation of Gram-positive Cocci; Culture Morphology

Examples of the colony morphology associated with each family is represented on this slide.

Micrococcus luteus, a commonly found member of the *Micrococcaceae*, produces round colonies which are convex (curving outward) with smooth, entire margins. Note the bright yellow, non-diffusable colony pigment which is a defining characteristic of *M. luteus*.

Next, we see an image of a mixed bacterial culture which displays the white, creamy, non-hemolytic colonies of *Staphylococcus epidermidis* mixed with the golden, hemolytic colonies of *Staphylococcus aureus*. The majority of staphylococcal species produce non-pigmented colonies with smooth margins, as opposed to the important pathogen, *Staphylococcus aureus*, which produces the characteristic colonies seen here.

The third image represents the colony morphology of many of the streptococci and enterococci. These families of bacteria produce colonies which are variable both in size, consistency, and the type of hemolysis produced on sBAP. Typical colonies are often described as gray to white, moist or glistening.

Slide 5: Hemolysis on sBAP

As mentioned, the type of hemolytic reaction produced on sBAP is a major clue toward the identification of the genus. Hemolysis is the lysis of the sheep erythrocytes within the agar by bacterial toxins (hemolysins) that are produced by the different genera of Gram-positive cocci.

Three types of reactions are observed:

1. Complete lysis of the erythrocytes in the vicinity of the growing colony. This is designated as beta (β)-hemolysis.
2. The partial decomposition of the hemoglobin of the erythrocytes by a weaker hemolysin. This is termed alpha (α) hemolysis and results in a greenish color surrounding the colony.
3. Gamma (γ) is the designation for the production of no hemolytic reaction.

Slide 6: Differentiation of Gram-positive Cocci; Hemolytic Reaction on sBAP

All three types of hemolytic reactions are represented on this slide. Notice that beta-hemolysis results in a total lysis or clearing of the agar in the area of colony growth. If the agar plate is held up to a light source, you can sometimes see through the agar, as is pictured on the left. Beta-hemolysis is a defining characteristic of *Staphylococcus aureus* and a certain streptococci.

Alpha-hemolytic colonies often assume a greenish hue. *Streptococcus pneumoniae* and a group of streptococci referred to as viridans (green) streptococci are examples of bacteria producing alpha-hemolysis. Gamma or no hemolysis results in no characteristic display in the vicinity of the colony.

Slide 7: Microscopic Morphology

After you have cultivated your organism on agar media, the next step is the microscopic examination. This technique is centered on the performance of the classic Gram's stain. The categorization of bacteria in the clinical laboratory is based on the reactivity of the organism when stained by the Gram's staining reagents. Due to the thick structure of the bacterial cell wall, Gram-positive cocci retain the primary Gram's stain, Crystal Violet. Gram-positive organisms appear purple under the microscope as opposed to the other category of bacterial cell with its own unique cell wall, the Gram-negative rod or coccus, which stains red.

Slide 8: Microscopic Morphology

When viewing the organism microscopically, the staining characteristics of the organism provide important information for identification. Each family of Gram-positive cocci has its own unique configuration. Streptococci and enterococci are observed to produce pairs and chains of cocci of varying length. The micrococci produce tetrads consisting of 4 cocci clustered together. The staphylococci are characterized by the formation of clusters of many cocci. The configurations are the result of the plane of cellular division exhibited by the organism.

Slide 9: Differentiation of Gram-positive Cocci; Gram Stain Morphology

The typical microscopic morphology of the Gram-positive cocci when using the Gram's stain is represented in these three images. The tetrads of the micrococci, the "grape" like clusters of the staphylococci, and the pairs and/or chains of the streptococci are shown.

Slide 10: Catalase Reaction

After observation of the colony and microscopic morphology, the production of catalase by the isolate is documented. This simple-to-perform test categorizes the Gram-positive cocci as either catalase producers (+) or non-producers (-). Catalase is an enzyme produced by the organism to neutralize the toxic effects of hydrogen peroxide in the environment of the cell. The laboratory assay is performed by mixing a sample of the bacterial colony with a drop(s) of hydrogen peroxide placed on a microscope slide. Catalase, if present, will break down the hydrogen peroxide to water and oxygen. Production of bubbles indicates a positive reaction. Care must be taken when using a sample of organism acquired from a sBAP since erythrocytes also produce catalase.

Slide 11: Differentiation of Gram-positive Cocci; Catalase Test

The bubbling reaction is almost immediate and distinct in appearance. The staphylococci and micrococci are catalase producers while the streptococci, enterococci, and a variety of other Gram-positive cocci are catalase-negative.

Slide 12: Differentiation within the Families of GPC

Once a presumptive identification has been made based on colony and microscopic morphology and the catalase reaction, additional tests can be performed to establish the genus and species of the organism. For the micrococci, susceptibility to the antimicrobial agents, bacitracin and furazolidone, as well as the modified oxidase test can be performed to distinguish this group from the staphylococci. A very important test in the categorization of the staphylococci is the coagulase test. Staphylococci are either producers of the enzyme coagulase or non-producers. The pathogen, *Staphylococcus aureus*, is notably coagulase-positive while most other members of the family are coagulase-negative. The streptococci and enterococci are categorized by expression of either beta, alpha, or gamma hemolysis on sBAP, depending on the genus/species. Some streptococci also possess unique cell wall carbohydrate antigens that can be identified by reactivity with specific indicator antibodies in an agglutination assay (Lancefield typing). Because of their diversity, there are a variety of biochemical tests that are used by laboratories to identify the Gram-positive cocci. Each genus lends itself to a separate tutorial. With the advent of newer molecular techniques, genus and species confirmation is even faster and easier.

Slide 13: Differentiation within Families of GPC

Members of the Staphylcocci can be differentiated based on production of the enzyme coagulase. Coagulase, which is an enzyme involved in the conversion of serum fibrinogen to fibrin, causes the clotting of plasma. Coagulase production is used as a virulence factor by the organism. All pathogenic strains of *S. aureus* are coagulase positive whereas the nonpathogenic species (*S. epidermidis* for example) are coagulase negative.

Slide 14: The Coagulase Test

The coagulase test is performed by mixing a sample of organism with rabbit plasma. Following incubation at 37^{o}C, if the plasma forms a clot, the organism is coagulase positive.

Slide 15: Differentiation of Gram-positive Cocci

A summary table is included which lists the results of the identification criteria discussed in the tutorial.

Slide 16: Miscellaneous GPC

Lastly, do not underestimate the variety of different genera in the four families of the Gram-positive cocci that can, under the right circumstances, move from relatively harmless saprophytes to disease-producing opportunists. Although the initial placement of the Gram-positive cocci into broad categories is relatively easy, further classification of some isolates can be challenging and requires a battery of tests to arrive at a definitive identification.

Slide 17: References

Slide 18: Disclosures

Slide 19: Thank You from www.TraineeCouncil.org
Thank you for joining me, Karen Krisher, on this Pearl of Laboratory Medicine on “Basics of Differentiation of Gram-positive Cocci.”