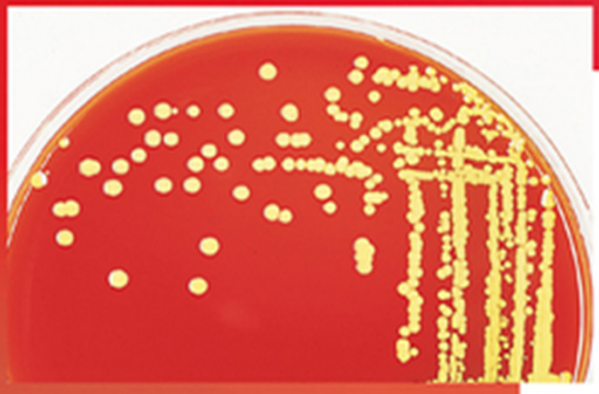
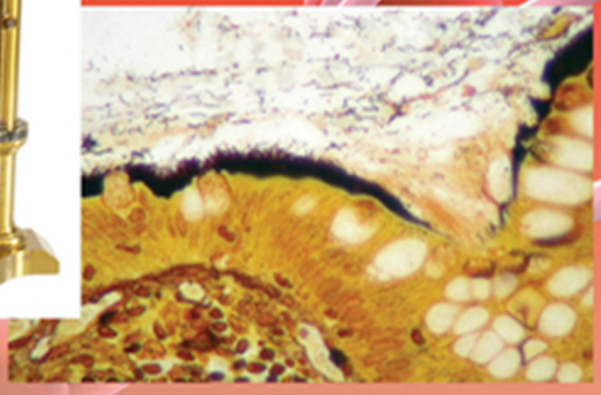
Luis M. de la Maza | Marie T. Pezzlo
Cassiana E. Bittencourt | Ellena M. Peterson

THIRD EDITION

Color Atlas of Medical Bacteriology





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Preface

At the dawn of the third millennium, when the names of Watson and Crick are more familiar to students than Koch and Pasteur, when the acronyms NAAT and MALDI-TOF are replacing hands-on identification methods, who needs an atlas of bacteriology?

Traditionally, diagnostic bacteriology has been in great part dependent on the subjective interpretation of a Gram stain or growth on an agar plate. While there are several excellent textbooks on the subject, they usually are written descriptions of microorganisms with few images. In part, we were motivated to publish the first edition of this atlas by the challenge to find illustrations for our own lectures and laboratory presentations. However, medical bacteriology is dynamic, with frequent changes especially in taxonomy and methodology. Therefore we accepted the challenge of an update.

In this third edition of *Color Atlas of Medical Bacteriology* we have included new illustrations of typical Gram stains, colony morphologies, and biochemical reactions and have added a large section on the histopathology of some organisms. We have also included a new chapter, addressing total laboratory automation, and the Fast Facts tables in chapter 42 which summarize critical details of the bacteria discussed in this third edition. "Total laboratory automation" implementation is so far limited to large-volume laboratories, but some automation components are already in use in medium-sized facilities. The purpose of the Fast Facts chapter is to help laboratorians, working on a clinical specimen, who may need a quick reminder of the characteristics of a particular bacterial isolate, and all students looking for a quick "refresher" in preparation for an exam.

Each book chapter has a brief introduction to provide context for the illustrations. For in-depth background of individual organisms, the reader should consult one of the many excellent textbooks and manuals available. This third edition was structured with reference to a number of sources, listed below, but in particular to the *Manual of Clinical Microbiology*, 12th edition (MCM12), from ASM Press. However, we are responsible for any errors that appear in this atlas. The number of images that we include of a particular organism does not necessarily correlate with the frequency of its isolation or its clinical relevance. Certain bacteria have variable, distinctive, or unique pictorial characteristics, and we have tried to provide a representative sampling of these. We hope you will find this atlas a useful reference tool.

The implementation of genomics and proteomics is revolutionizing diagnostic clinical microbiology. However, like Janus, all revolutions have two faces. On the positive side, these new approaches have already helped the clinical laboratory to significantly improve the sensitivity and specificity of the identification of many microorganisms. These methodologies have also expedited organism identification, thus improving patient management. Significant challenges are still ahead before we can take full advantage of these new technologies. The extraordinary complexity of the human microbiome is going to require the deployment of massive resources before we can collect, classify, and interpret the data. However, before we get there, we are going to have to learn how to deal with the second face of Janus. Although changes in taxonomy may have very positive effects on medical practice by guiding more specific treatments, they also result in practical problems for the microbiology laboratory on how to incorporate these changes into their practice while maintaining clinician satisfaction and, at the same time, preventing potential negative outcomes. There is an urgent need to establish clear guidelines for defining new families, genera, and species of microorganisms. These guidelines should be written by a group of individuals who represent various areas of expertise including taxonomy, biology, and health sciences.

With the increasing use of genomics and proteomics, the remarkable forms, shapes, and colors of bacteria in the laboratory are rapidly being replaced by signals only measurable by instruments. The time is very near when we will be showing our grandchildren many of the images in this atlas that have become a distant memory. In the meantime, let us enjoy the beauty of the colorful bacterial world.

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Technical Note

The microscopic pictures were taken with a Zeiss Universal microscope (Carl Zeiss, Inc., West Germany) equipped with Zeiss and Olympus (Olympus Optical Corp., Ltd., Japan) lenses. The majority of histopathological images were obtained either with a Nikon Eclipse Ci microscope, Nikon lenses, and Nikon eyepieces CFI 10x/22 equipped with a digital Nikon DS-Fi1 (Nikon Corporation, Japan) camera or with a Nikon Eclipse 50i microscope, Nikon lenses, and Nikon eyepieces CFI 10x/22 equipped with a digital Nikon Coolpix 4500 camera. The final magnification of the Gram and acid-fast stains is ×1,200.

Most of the macroscopic images were captured with a Contax RTS camera with a Carl Zeiss S-Planar 60 mm f/2.8 lens and a Nikon EL camera with a Micro-Nikkor 55 mm f/3.5 lens. An Olympus SP-800UZ 14MP digital camera with an ED lens 30× wide optical 4.9–147 mm 1:2.8–5.6 was used for a few images.

Provia 100F and 400F Professional Fujichrome film (Fuji Photo Film Co., Ltd., Tokyo, Japan) and Kodachrome 25 Professional film (Eastman Kodak Co., Rochester, NY) were used with the analog equipment.

About the Authors

Luis M. de la Maza

A native of Spain, Luis de la Maza obtained his MD degree from the University of Madrid and his PhD from the University of Minnesota. After completing his residency training program in Pathology and Laboratory Medicine in Boston and Minnesota, he spent four years at NIH characterizing the molecular structure and biology of adeno-associated virus. In 1979 he became the Medical Director of the Division of Medical Microbiology at the University of California, Irvine School of Medicine, where he is also the Medical Director of the Clinical Laboratory Scientist training program. His research is focused on the formulation of a *Chlamydia trachomatis* vaccine.

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Marie Pezzlo received her Bachelor and Master of Science degrees and medical technologist certification at the University of Connecticut and St. Joseph College. Her passion for clinical microbiology began while working at Hartford Hospital under the directorship of Dr. Raymond Bartlett. Eight years later she became the Senior Supervisor of the Medical Microbiology Division at the University of California, Irvine Medical Center. Her research interest has been focused on rapid detection of microorganisms, especially in urinary tract infections. Throughout her career she has been an active member of the American Society for Microbiology, volunteering in many activities of the Society.

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A native of Brazil, Cassiana Bittencourt obtained her MD degree from the Metropolitan University of Santos. She completed her pathology residency at University of South Florida and a fellowship in microbiology at University of Texas Southwestern Medical Center. She is certified by the American Board of Pathology in Anatomic and Clinical Pathology as well as Medical Microbiology. In 2016, she joined the Department of Pathology at the University of California, Irvine School of Medicine as Medical Director of the Division of Medical Microbiology. Her current interests include infectious disease histology, application of non-culture-based methods, and resident education.

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Ellena Peterson received her undergraduate degree from the University of San Francisco and her PhD in Microbiology and Immunology from Georgetown University. In 1978 she joined the Department of Pathology at the University of California, Irvine School of Medicine. During her tenure she served as the Associate Dean of Admissions for the School of Medicine and as the Associate Director of the Clinical Microbiology Laboratory, and presently she is the Program Director of the Clinical Laboratory Scientist Program. Her research has been focused on the pathogenicity of *Chlamydia* spp.

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Staphylococcus, *Micrococcus*, and Other Catalase-Positive Cocci

1

Members of the genera *Staphylococcus, Micrococcus*, *Kocuria*, and *Kytococcus* are characterized as being catalase-positive, Gram-positive cocci that occur in pairs and clusters. These organisms commonly colonize the surfaces of skin and mucosal membranes of mammals and birds. Members of the genus *Staphylococcus* are important human pathogens, whereas the other genera in this chapter play a lesser role in human infections and thus are discussed separately.

Traditionally, members of the genus *Staphylococcus* have been divided into those that are coagulase positive, i.e., *Staphylococcus aureus*, and those that are referred to as coagulase-negative staphylococci (CoNS), i.e., all others, based on their ability to clot rabbit plasma. The species of *Staphylococcus* most frequently associated with human infections is *S. aureus*, which is a major cause of morbidity and mortality. *S. aureus* can produce disease mediated by toxins or by direct invasion and destruction of tissues. *S. aureus* infections range from superficial skin infections to fatal systemic infections that can occur when the integrity of the skin is damaged, thus giving this pathogen access to sterile sites. Among the more common *S. aureus* infections are boils, folliculitis, cellulitis, and impetigo. Immunocompromised hosts are at particular risk of infection. Systemic infections include septicemia, which can result in the seeding of distant sites, producing osteomyelitis, pneumonia, and endocarditis. Toxigenic strains of *S. aureus* are capable of producing bullous impetigo, scalded-skin syndrome, and toxic shock syndrome. *S. aureus* is also a well-known contributor to food poisoning due to the elaboration of enterotoxins in foods such as potato salad, ice cream, and custards. Intense vomiting and diarrhea usually occur within 2 to 8 h after ingestion of food containing the toxin.

CoNS, in particular *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus haemolyticus*, *Staphylococcus lugdunensis*, and *Staphylococcus schleiferi*, play a role in human infection. In particular, *S. epidermidis* is recognized as a leading cause of health care-related infections, with immunocompromised hosts being at increased risk. Because CoNS are members of the normal skin and mucosal membrane microbiota, they are frequently considered a contaminant when isolated from clinical specimens and therefore may be overlooked as a cause of infection. This is compounded by the fact that their clinical presentation is subacute, unlike that of *S. aureus*. An important virulence property of CoNS is their ability to form a biofilm on the surface of indwelling or implanted devices, making them frequent agents of intravascular infections. *S. epidermidis* has also been implicated as a cause of endocarditis and is associated with right-side endocarditis in intravenous-drug users. *S. saprophyticus* is a leading cause of noncomplicated urinary tract infections in young, sexually active females, second only to *Escherichia coli* in this patient population. Of the more recently described CoNS human pathogens, *S. lugdunensis* and *S. schleiferi* have been implicated in serious infections, including endocarditis, septicemia, arthritis, and joint infections. *S. lugdunensis*, which more frequently colonizes skin and infects tissue

(causing, e.g., boils and abscesses) below the waist, at times can behave more like *S. aureus* than CoNS. This organism has been associated with aggressive infections, such as endocarditis, that have a high mortality rate; therefore, rapid recognition of this species is important for initiation of appropriate antimicrobial therapy. While other species of CoNS have been implicated in a variety of infections, they occur with less frequency.

An increasing problem with *S. aureus* and CoNS is resistance to antimicrobial agents, in particular methicillin. In the majority of methicillin-resistant *S. aureus* (MRSA) strains, this is due to production of an altered penicillin-binding protein, mainly PBP2a or PBP2c, encoded by the *mecA* or *mecC* gene, respectively, which is carried on a mobile genetic unit referred to as SCC*mec*. Overproduction of β-lactamase accounts for a smaller percentage of MRSA or MR-CoNS strains. In recent years, *S. aureus* strains with decreased susceptibility to vancomycin have been identified. These strains are referred to as vancomycin-intermediate *S. aureus* (VISA), as vancomycin-resistant *S. aureus* (VRSA) when the vancomycin MIC is ≥16 μg/ml, or, when their susceptibility to the glycopeptide class of antimicrobials as a whole is being addressed, as glycopeptide-intermediate *S. aureus* (GISA). Although only a few of these strains have been isolated, they pose a potential threat to effective treatment of serious *S. aureus* infections.

Testing for MRSA can be difficult due to heteroresistance, in which the resistance is expressed to a different extent among subpopulations. In susceptibility tests performed by disk diffusion or tests to determine a MIC, cefoxitin has been shown to have greater sensitivity for detecting MRSA than oxacillin, also a common antibiotic used to test for MRSA. Molecular assays to directly detect the *mecA* gene, as well as rapid assay formats employing monoclonal antibodies to the altered PBP2a protein, have been used to circumvent the problems of *in vitro* susceptibility testing for MRSA. In addition, due to the importance of rapidly identifying cultures positive for *S. aureus*, in particular MRSA, primers and probes for *S. aureus* and MRSA have been incorporated into a number of nucleic acid-based panels and individual nucleic acid amplification assays. Depending on the assay, these tests can be performed directly from clinical specimens or on blood cultures positive for Gram-positive cocci in pairs and clusters. In addition, screening for MRSA from nares cultures can be done by nucleic acid amplification or use of selective chromogenic agars. Identifying VISA strains by standard susceptibility methods remains a challenge; however, VRSA strains with higher vancomycin MICs can be identified using broth dilution, select automated systems, and screening agar incorporating 6 μg/ml of vancomycin.

Upon incubation in air at 35°C for 24 to 48 h, staphylococci grow rapidly on a variety of media, with colonies that range from 1 to 3 mm in diameter. On blood agar, staphylococci produce white to cream, opaque colonies. *S. aureus* colonies typically are cream in color but occasionally have a yellow or golden pigment, a phenotypic characteristic that led to the species name. *S. aureus* can be beta-hemolytic, and it is not uncommon to see both large and small colonies in the same culture, a phenotypic characteristic shared by several heteroresistant MRSA strains. CoNS, especially *S. epidermidis*, produce white colonies; however, other CoNS strains and species can have colonies with a slight cream pigment. In general, CoNS strains are nonhemolytic; however, some produce a small zone of beta-hemolysis on blood agar.

Since *S. aureus* is frequently isolated in mixed cultures, selective and differential media are used to facilitate the detection of these organisms in clinical material, particularly in nasal swabs, which are used to screen for carriage of this bacterium. Mannitol salt agar is an example of this, where the high concentration of salt (7.5%) inhibits many other organisms. Mannitol, along with the phenol red indicator in the medium, facilitates the discrimination of *S. aureus*, which can ferment mannitol, from most CoNS. However, since other organisms can grow on this medium and strains of CoNS can also ferment mannitol, additional testing is required. As mentioned above, chromogenic media selective and differential for MRSA are more commonly used for screening nasal cultures.

In addition to their distinctive Gram stain morphology (Gram-positive cocci in pairs and clusters), a common characteristic of these organisms is that they are catalase positive. The coagulase test, which measures the ability to clot plasma by converting fibrinogen to fibrin, is useful in distinguishing *S. aureus* from other bacteria that appear similar. A suspension of the organism to be identified is inoculated into rabbit plasma containing EDTA and incubated at 35°C for 4 h. The tube is tilted gently, and the presence or absence of clot formation is noted. If the test is negative at 4 h, the suspension is incubated for up to 24 h. The 4-h reading is important because some strains

produce fibrinolysin, which can dissolve a clot upon prolonged incubation, causing a false-negative result. Some strains of MRSA produce a very weak coagulase reaction, resulting in a negative reading. Bound coagulase (clumping factor) can be detected by a slide agglutination test, in which a suspension of the organism is emulsified on a slide with a drop of rabbit plasma. If bound coagulase is present, the organisms agglutinate. For correct interpretation of this test, a control in which saline is used instead of plasma is needed to check for autoagglutination. Of the CoNS, *S. lugdunensis* and *S. schleiferi* can also test positive for bound coagulase but can be differentiated from *S. aureus* by a negative tube coagulation test. Alternatively, commercially available tests can be used that are based on latex particles that have been coated with plasma, immunoglobulin, or (in some versions of this test) antibodies to the more common polysaccharide antigens. The plasma detects bound clumping factor, while the immunoglobulin binds protein A and the antibody to the polysaccharide antigens binds serotype antigens present on the surface of *S. aureus*. Some strains of MRSA, however, may be negative by this method because of low levels of bound coagulase and protein A, and false-positive reactions can occur due to the presence of the polysaccharide antigens present on some CoNS isolates.

Strains of *S. aureus* that produce a weak coagulase reaction can be further tested by the DNase test or a thermostable-endonuclease test. *S. aureus* and *S. schleiferi* possess enzymes that can degrade DNA, a DNase and a thermostable endonuclease. Both tests use the same basic medium containing agar that incorporates DNA and the metachromatic dye toluidine blue O. A heavy suspension of organisms is spotted onto the plate; after 24 h of incubation at 35°C, a pink haze appears around the colony, in contrast to the azure blue of the medium. In tests for the thermostable endonuclease, a suspension of the organism is boiled before being placed on the DNA plate.

CoNS can be identified to the species level based on their susceptibility profiles in response to selected agents, most notably novobiocin, as well as key biochemicals. A variety of commercial systems combine several biochemical tests to allow differentiation among the CoNS. While most of the CoNS of clinical importance are novobiocin susceptible, *S. saprophyticus* is novobiocin resistant. Other tests that can be used to differentiate among the species are those for phosphatase activity, production of acetoin, polymyxin susceptibility, pyrrolidonyl arylamidase activity, and acid production from carbohydrates.

While biochemical tests are still commonly used to identify strains of *Staphylococcus* to the species level, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry is rapidly replacing traditional biochemical algorithms.

Several species that once were in the genus *Micrococcus* and that have been reported to play a role in human infections have been reclassified into the genera *Kocuria* and *Kytococcus*. Despite the reclassification, collectively these organisms are often referred to as micrococci. Members of the micrococci have a higher G+C content than the staphylococci. They are also common inhabitants of the skin but have a fairly low pathogenic potential. However, infections with these organisms have occurred in immunocompromised hosts. *Micrococcus luteus* and related organisms have been implicated in a variety of infections, including meningitis, central nervous system shunt infections, endocarditis, and septic arthritis.

Micrococci, in addition to forming pairs and clusters, can appear as tetrads. Like the staphylococci, they can be easily grown in the laboratory and can be recovered from a variety of media. However, in comparison to staphylococci, they are slower growing, with smaller colonies present after 24 h of incubation at 35°C. In addition, depending on the species, the colony color can range from cream to yellow (*M. luteus*) or rose red. As with CoNS, a variety of commercial systems that incorporate several tests, including urease, acid production from carbohydrates, esculin, and gelatin, have been employed to aid in the differentiation of this group. Bacitracin, lysostaphin, and furazolidone have been used to aid in differentiating staphylococci from micrococci. In general, staphylococci are resistant to bacitracin (0.04-U disk), in contrast to micrococci, which are susceptible, while the opposite is found with furazolidone (100-μg disk) and lysostaphin (200-μg disk), where micrococci are resistant. MALDI-TOF has aided in the identification of micrococci, and with more strains being added to present databases, this method is rapidly becoming the method of choice for identification of micrococci.

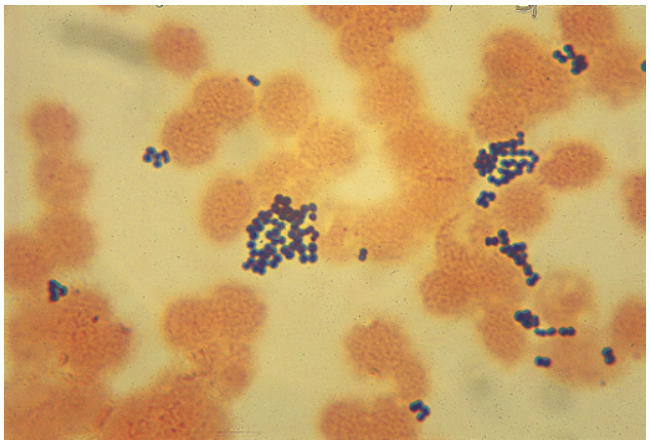


Figure 1-1 Gram stain of *Staphylococcus aureus*. A Gram stain of a positive blood culture shows Gram-positive cocci in grape-like clusters. On subculture to solid medium, *S. aureus* was isolated.

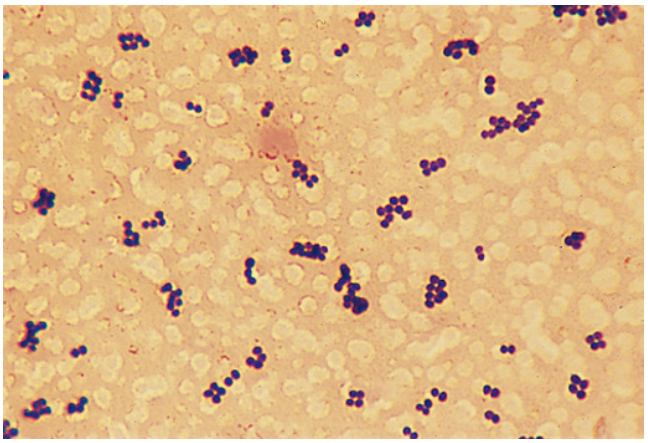


Figure 1-2 Gram stain of *Micrococcus luteus*. *M. luteus* is a Gram-positive coccus that, like *S. aureus*, can appear in pairs and clusters. However, it also tends to form tetrads, as depicted in this Gram stain.

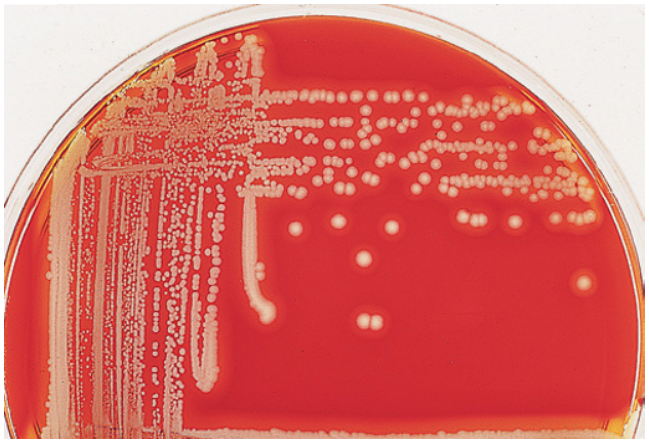


Figure 1-3 *Staphylococcus aureus* on blood agar. Shown in this image is a culture of *S. aureus* grown overnight at 35°C on blood agar. The colonies are cream colored and opaque and have a smooth, entire edge. A zone of beta-hemolysis surrounds the colony.

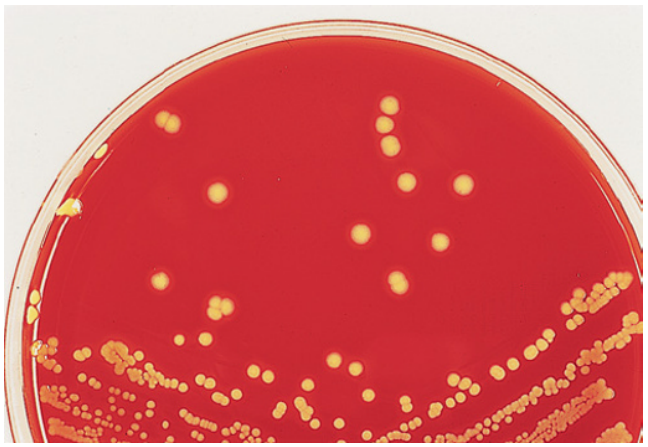


Figure 1-4 Golden pigment of *Staphylococcus aureus*. *S. aureus* is capable of producing the golden pigment that led to its species name. In practice, strains with this degree of pigment are not frequently isolated from clinical specimens. The isolate shown was incubated overnight on blood agar at 35°C and then left at room temperature for an additional day. When left at room temperature or refrigerated following incubation, isolates tend to develop more intense pigment.

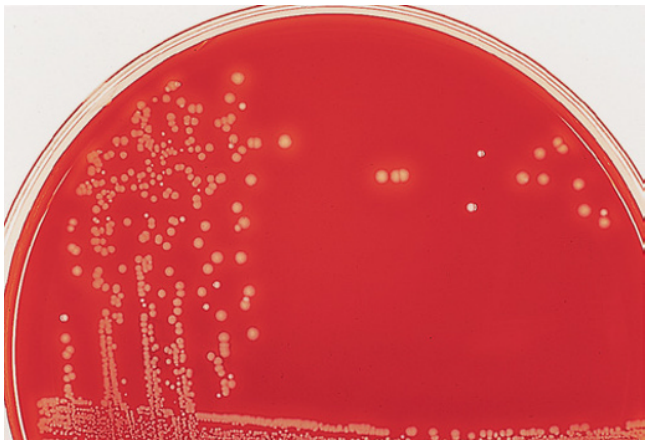


Figure 1-5 Size variation of *Staphylococcus aureus* colonies. It is not uncommon for strains of *S. aureus*, especially MRSA strains, to produce colonies that are heterogeneous in size and the degree of hemolysis. Colonies shown were grown on blood agar for 24 h at 35°C.

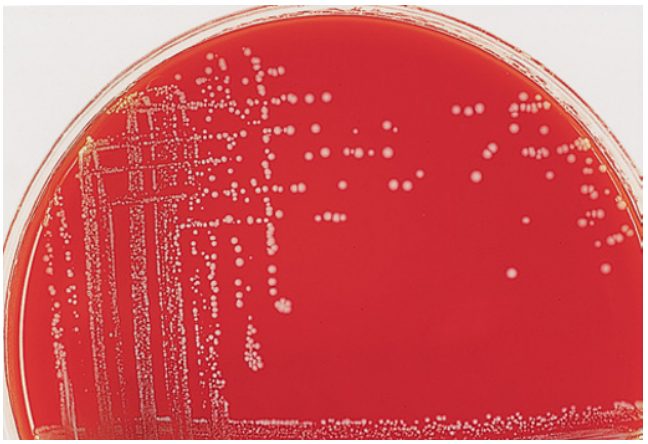


Figure 1-6 *Staphylococcus epidermidis* on blood agar. *S. epidermidis*, in contrast to both *S. aureus* and other CoNS, produces a white colony with little or no pigment. The isolate shown here was grown on blood agar for 24 h at 35°C. This strain of *S. epidermidis* also exhibits some variation in colony size.

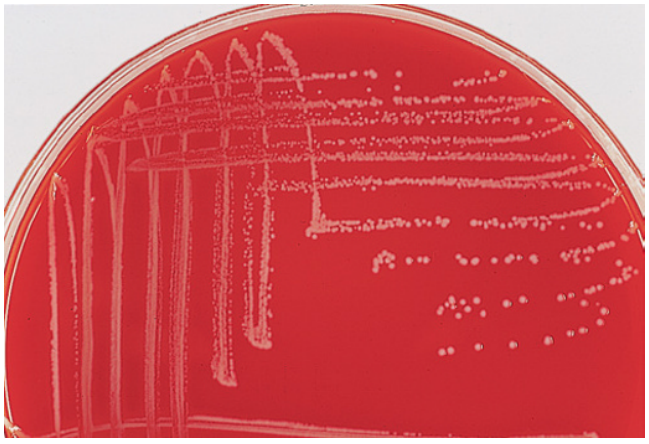


Figure 1-7 *Staphylococcus lugdunensis* on blood agar. Colonies of *S. lugdunensis* on blood agar resemble *S. epidermidis* colonies; however, they tend to be cream colored, in contrast to the typical white colonies of *S. epidermidis* (Fig. 1-6).

Figure 1-8 *Micrococcus luteus* on blood agar. A distinguishing feature of *M. luteus* is the vivid yellow colonies it produces. The isolate shown here was grown on blood agar for 72 h at 35°C. In general, *Micrococcus* is slower growing than *Staphylococcus*.

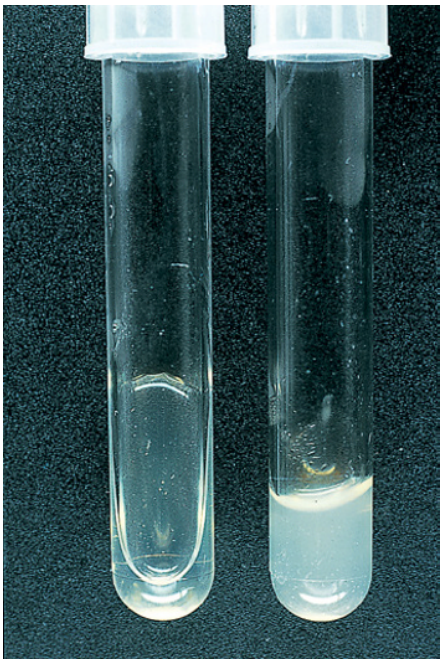


Figure 1-9 Coagulase test. A common method used to distinguish *S. aureus* from other *Staphylococcus* spp. is the tube coagulase test shown here. *S. aureus* is positive, and CoNS are negative. Colonies of the isolate to be identified were emulsified in 0.5 ml of rabbit plasma. The tube was incubated at 35°C for 4 h and tipped gently to look for clot formation. The tube on the left is negative, with the plasma remaining liquid, while the tube on the right is positive, as evidenced by the clot formation. Tubes giving negative results at 4 h should be incubated for up to 24 h.

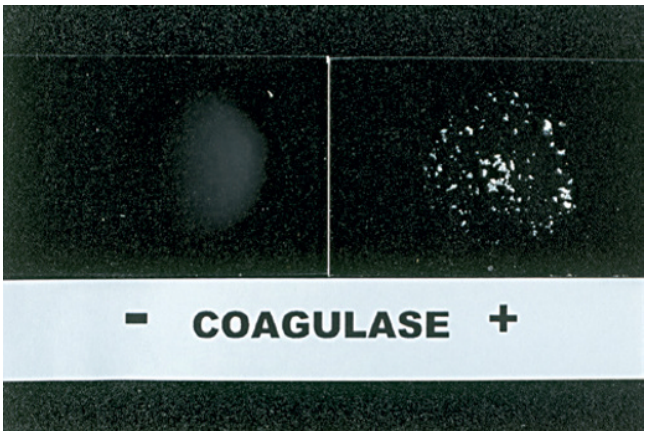


Figure 1-10 Slide coagulase test. The slide coagulase test is a rapid assay that tests for clumping factor on the surface of the organism. The test is performed by emulsifying the organism to be identified in both saline, which serves as a control for autoagglutination (left), and rabbit plasma (right). Agglutination of the organisms only in plasma is a positive result. *S. aureus* (right) is positive by this test as shown in this figure, as are strains of *S. lugdunensis* and *S. schleiferi*.

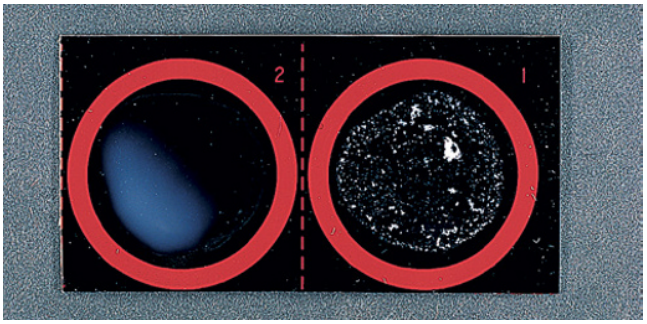


Figure 1-11 Latex test for the identification of *Staphylococcus aureus*. In the test depicted here, latex particles have been coated with antibody that can recognize bound coagulase as well as immunoglobulin that will bind to protein A present on the surface of most strains of *S. aureus*. *S. epidermidis* (left), which serves as a negative control, and the isolate to be identified (right) were emulsified with coated latex beads. The isolate shown here was identified as *S. aureus*. As with the slide coagulase test, some strains of MRSA may be negative and some strains of CoNS, namely, *S. lugdunensis* and *S. schleiferi* strains, may be positive.

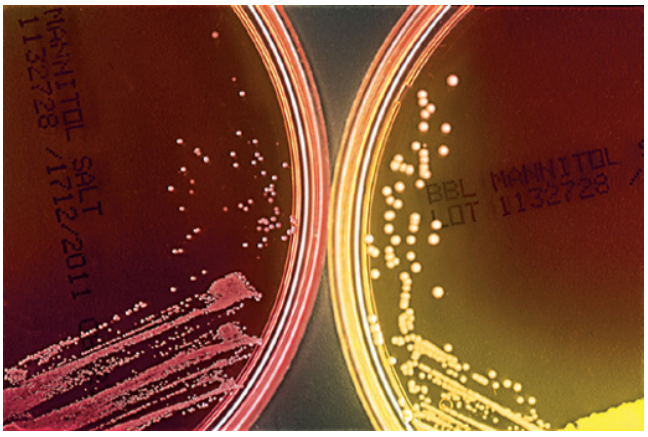


Figure 1-12 Mannitol salt agar. Mannitol salt agar is a selective and differential medium used for the isolation and presumptive identification of *S. aureus*. The high salt concentration inhibits the growth of many organisms that inhabit skin and mucosal membranes. The phenol red indicator incorporated into the medium detects acid production (yellow) resulting from the fermentation of mannitol. Here, CoNS (left) and *S. aureus* (right) were inoculated on the agar and then were incubated overnight.

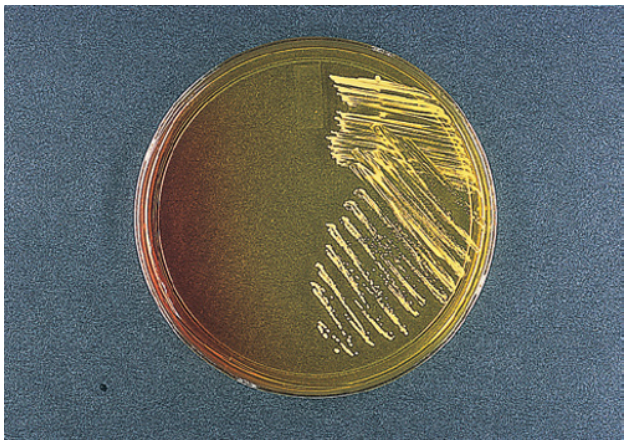


Figure 1-13 Mannitol salt agar containing oxacillin. Mannitol salt agar with oxacillin can be used to screen for the presence of MRSA in nasal specimens, since the 7.5% salt and 6 µg of oxacillin in this medium inhibit most other organisms that normally colonize the nares. MRSA turns the medium yellow as a result of the fermentation of mannitol. Pictured here is a plate inoculated with a methicillin-susceptible *S. aureus* strain (left) and a MRSA strain (right). The methicillin-susceptible *S. aureus* strain failed to grow. As with most *in vitro* testing for methicillin susceptibility, oxacillin (not methicillin) is used because of its higher stability.

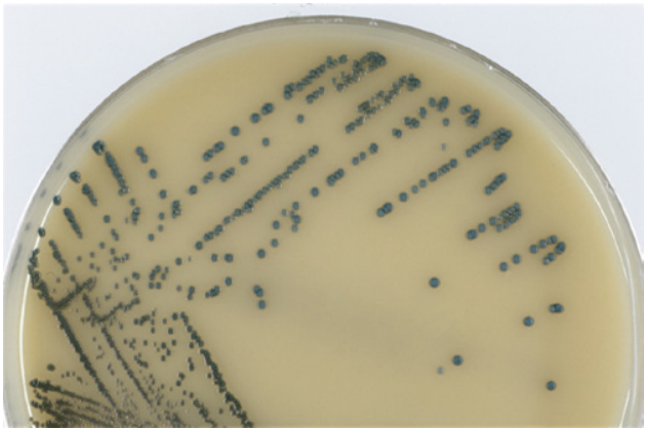
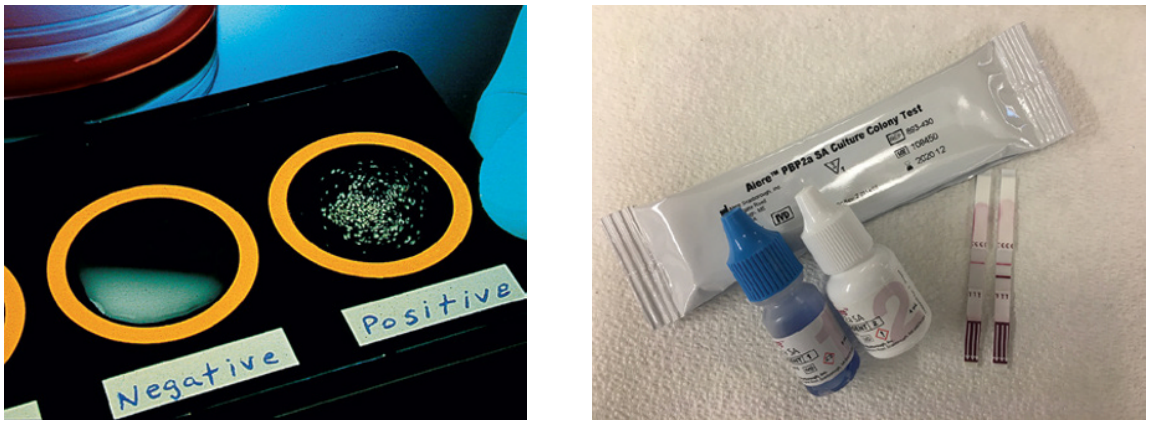


Figure 1-14 Spectra MRSA. Shown is a chromogenic medium used to detect MRSA, Spectra MRSA (Thermo Scientific, Remel Products, Lenexa, KS), which is both selective and differential. When the chromogenic substrate incorporated into the inhibitory agar is degraded by the enzymatic action of MRSA, the colony takes on a denim blue color. Shown here is an overnight nasal culture from which MRSA was isolated.



A B

Figure 1-15 Assays to detect PBP2a found in MRSA. (A) The product of the *mecA* gene, which results in methicillin resistance, is an altered penicillin-binding protein, PBP2a. Monoclonal antibody to this altered protein was used to coat latex particles, which were then used in the Oxoid agglutination assay to detect PBP2a. (B) Shown is the Alere PBP2a SA Culture Colony Test (Alere Scarborough, Inc., Scarborough, ME), a lateral flow assay that utilizes monoclonal antibodies for the detection of PBP2a. Both formats are rapid tests that are used once the organism is isolated on solid medium.

Figure 1-16 DNase plate to differentiate *Staphylococcus aureus* from CoNS. *S. aureus* produces DNase, which can degrade DNA. This property is used to aid in the differentiation of CoNS (left) from *S. aureus* (right). This is particularly useful for identification of *S. aureus* strains that produce a small amount of coagulase, thus giving an equivocal or weakly positive coagulase test. The only CoNS species that shares this property with *S. aureus* is *S. schleiferi*. In this test, a heavy inoculum of the organism is used to spot an agar plate that contains DNA and toluidine blue. If the organism produces DNase (right), the DNA is degraded, resulting in the agar turning pink in the area surrounding the inoculum due to the metachromatic qualities of toluidine blue.

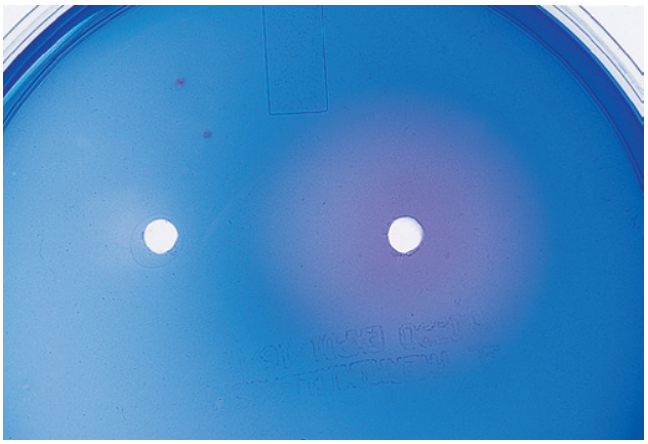


Figure 1-17 Thermostable endonuclease activity. In addition to DNase, *S. aureus* produces a thermostable endonuclease that can also cleave DNA. To test for this activity, a heavy suspension of the organism is boiled and then used to fill a well that is cut in the DNA plate containing toluidine blue. As described in the legend to Fig. 1-16, if the DNA is degraded, there is a change in the color of the agar from blue to pink. *S. epidermidis* does not produce a heat-stable endonuclease (left), whereas the *S. aureus* strain (right) does, as shown by the pink zone around the well containing *S. aureus*.

Figure 1-18 Ornithine decarboxylase test for the identification of *Staphylococcus lugdunensis*. Unlike most other CoNS species, *S. lugdunensis* is ornithine decarboxylase positive. Decarboxylase medium containing 1% ornithine is inoculated and incubated overnight. Since some strains of *S. epidermidis* can also be positive at 24 h, the specimen should be examined at 8 h, a time at which *S. lugdunensis* is positive but *S. epidermidis* is still negative. The isolate on the left, *S. saprophyticus*, is negative since it is yellow, indicating only fermentation of glucose; however, *S. lugdunensis* (right) is positive, as shown by the rose color resulting from the alkalinization of the medium.

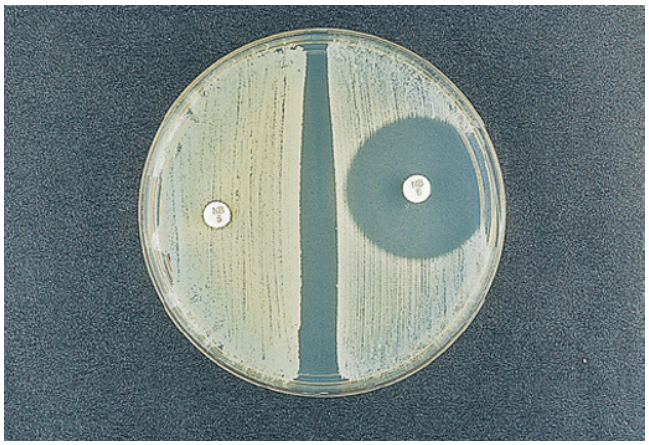


Figure 1-19 Novobiocin susceptibility. *S. saprophyticus* can be differentiated from other clinically significant CoNS isolates by its resistance to the antibiotic novobiocin. As pictured, Mueller-Hinton agar was inoculated with suspensions equivalent to a 0.5 McFarland standard of *S. saprophyticus* (left) and *S. epidermidis* (right). Novobiocin disks (5 μg) were placed on the agar surface, which was incubated for 24 h at 35°C. Zones of inhibition measuring ≤16 mm indicate novobiocin resistance, as seen with this isolate of *S. saprophyticus*, which has no zone of inhibition. In contrast, the susceptible *S. epidermidis* isolate has a large zone of inhibition around the novobiocin disk.

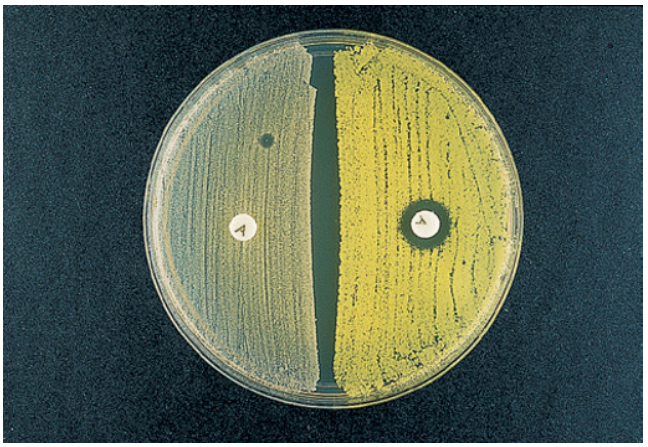
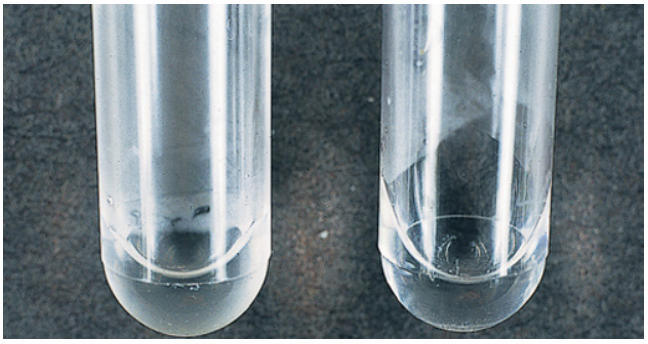


Figure 1-20 Bacitracin susceptibility. The same procedure used to test for the bacitracin susceptibility of *Streptococcus pyogenes* can be used to differentiate staphylococci, which are bacitracin resistant, from micrococci, which are susceptible. Here, *S. epidermidis* (left) is not inhibited, as shown by growth up to the disk containing 0.04 U of bacitracin, whereas *M. luteus* (right) exhibits a zone of inhibition around the bacitracin disk.

Figure 1-21 Lysostaphin susceptibility. Several species of *Staphylococcus* are susceptible to the endopeptidase lysostaphin, which cleaves the glycine-rich pentapeptide that is essential for cross bridging the cell wall. Cleavage of these basic units weakens the cell wall, making it susceptible to lysis. Depending on the makeup of this pentapeptide, specifically the glycine content, susceptibility to lysostaphin can vary. For example, while *S. aureus* is very susceptible, *S. saprophyticus* is less susceptible due to the serine content of its pentapeptide bridge. Micrococci are not susceptible to lysostaphin. As shown here, the test is performed by making a heavy suspension of the unknown organism in saline and then adding an equal volume of lysostaphin reagent. Clearing of the suspension after 2 h at 35°C indicates lysis of the organisms. In the example shown here, the lysostaphin test medium inoculated with *M. luteus* (left) remained turbid and thus was negative, in contrast to the tube on the right, which was inoculated with *S. aureus* and is positive, as shown by clearing or lysis of the bacterial suspension. This assay can also be performed as a disk diffusion test.



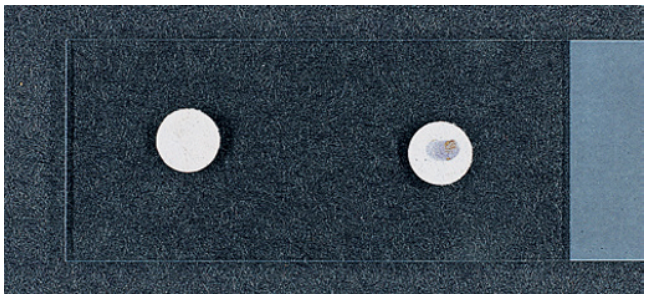


Figure 1-22 Modified oxidase test. A modified oxidase test, the Microdase test (Thermo Scientific, Remel Products), is available for differentiating micrococci from staphylococci. Micrococci possess cytochrome *c*, which is essential for producing a positive oxidase reaction, whereas clinically relevant staphylococci are oxidase negative, since they lack cytochrome *c*. In the example shown, a colony of *S. epidermidis* (left) and a colony of *M. luteus* (right) were rubbed onto a disk impregnated with tetramethyl-*p*-phenylenediamine (TMPD) dissolved in dimethyl sulfoxide. Development of a purple-blue color within 2 min indicates a positive test due to the reaction of the enzyme oxidase with cytochrome *c* and TMPD.

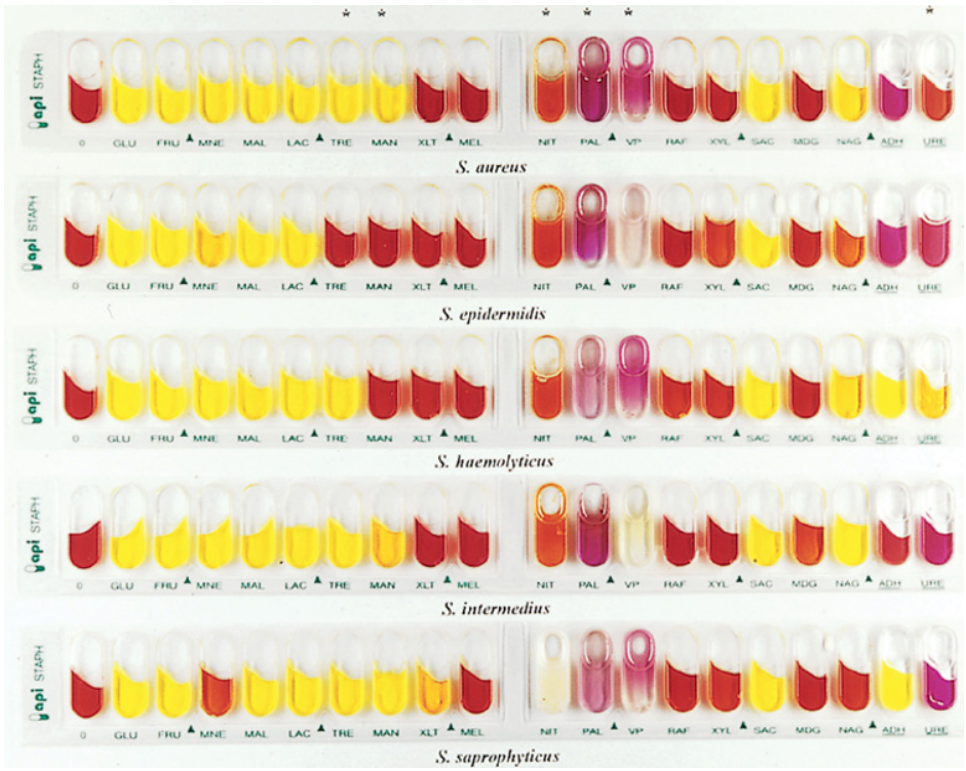


Figure 1-23 API Staph identification system. API Staph (bioMérieux, Inc., Durham, NC) is a commercial system that can differentiate among several *Staphylococcus* species. Each test strip consists of 20 microtubes, including the negative control well. Key reactions that aid in the differentiation and identification of the five *Staphylococcus* species shown are indicated by asterisks at the top.

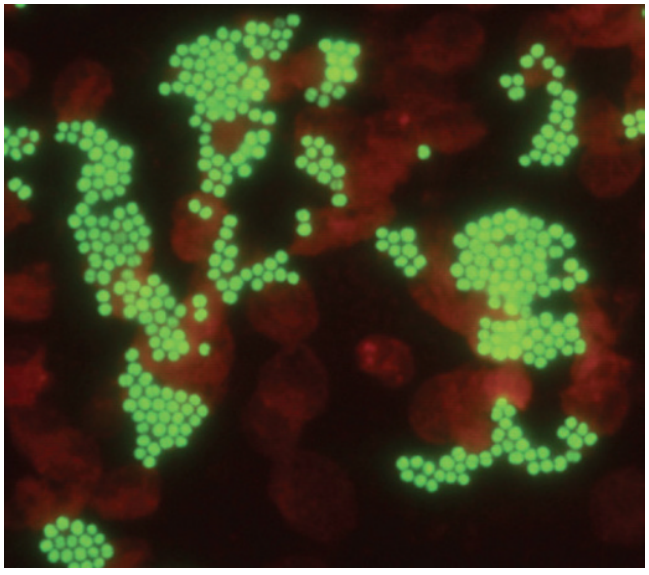


Figure 1-24 PNA FISH for the differentiation of *Staphylococcus aureus* from CoNS in blood cultures. PNA FISH (AdvanDx, Woburn, MA) is a 90-min fluorescent *in situ* hybridization (FISH) assay utilizing fluorescence-labeled peptide nucleic acid (PNA). It is performed directly from blood culture bottles that are positive for Gram-positive cocci in clusters. In the example shown, the Gram-positive cocci are *S. aureus*, which hybridized with a green fluorescent probe. (Courtesy of AdvanDx.)

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Streptococcus 2

The genus *Streptococcus* is composed of over 100 species and subspecies which are a predominant component of the normal bacterial microbiota of the respiratory, gastrointestinal, and genital tracts of humans. Members of this genus are Gram-positive cocci that occur in pairs and/or chains and are catalase-negative, facultative anaerobes that metabolize carbohydrates by fermentation, producing mainly lactic acid.

Traditionally, streptococci have been grouped by the phenotypic characteristics of hemolysis and Lancefield antigen composition as well as by pathogenic potential. While these are still useful ways to group organisms, there are many exceptions and overlapping characteristics with each grouping system; therefore, genetic analysis is a more definitive method for classifying these organisms. However, from a practical standpoint, phenotypic characteristics are very useful in identification algorithms. Streptococci can be either beta-hemolytic (complete hemolysis), alpha-hemolytic (incomplete hemolysis resulting in a green zone around the colony), or gamma-hemolytic (no hemolysis) on blood agar. With the Lancefield system, depending on the cell wall carbohydrate (Lancefield antigen) or lipoteichoic acid (group D), some of the streptococci have been placed in groups A, B, C, D, F, and G. Most members of the Lancefield groups are beta-hemolytic, the exception being group D, which is composed of alpha-hemolytic or nonhemolytic organisms.

STREPTOCOCCUS PYOGENES (GROUP A BETA-HEMOLYTIC STREPTOCOCCI)

Streptococcus pyogenes organisms are beta-hemolytic streptococci possessing the group A Lancefield antigen. *S. pyogenes* is one of the more virulent *Streptococcus* species and is responsible for a wide range of clinical entities, including pharyngitis, impetigo, bacteremia, and soft tissue infections. Sequelae resulting from infection include rheumatic fever, glomerulonephritis, the scarlatiniform rash of scarlet fever, toxic shock-like syndrome, and necrotizing fasciitis.

Direct detection of *S. pyogenes* based on the Lancefield group A antigen is commonly performed on throat specimens. There are several commercial kits available for direct detection of this antigen that have high specificity but vary in sensitivity. Therefore, especially with children, for whom the incidence of infection and potential to develop sequelae are greater, it is recommended that negative direct antigen tests be followed up by culture. Alternatively, direct detection of *S. pyogenes* by DNA amplification methods is more sensitive than direct antigen detection. Since, in contrast to culture, the sensitivity of some DNA-based amplification assays is >90%, culture confirmation is not required for negative amplification results. For culture, *S. pyogenes* is commonly isolated using blood agar incubated in 5% CO_2. Trimethoprim-sulfamethoxazole can be incorporated into the blood

agar to increase the selection of *S. pyogenes*. Antibodies specific for the group A Lancefield carbohydrate antigen can be used to identify *S. pyogenes*. Bacitracin susceptibility has also traditionally been used to identify large-colony beta-hemolytic organisms. Here, a 0.04-U bacitracin disk is applied to a lawn of the organism, and the formation of any zone of inhibition is considered a positive result. Another useful rapid biochemical test for identification of *S. pyogenes* is the PYR test, in which the enzyme pyrrolidonyl arylamidase is detected. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has also been shown to be useful in identifying *S. pyogenes*.

Small-colony beta-hemolytic organisms can also possess the Lancefield group A antigen; however, these isolates are classified as members of the viridans group streptococci, anginosus group. Small-colony (*Streptococcus anginosus*) and large-colony (*S. pyogenes*) group A streptococci can be differentiated biochemically in that *S. pyogenes* is PYR positive and Voges-Proskauer test (VP) negative, while the opposite is true of *S. anginosus*.

Serological tests are available to detect the host response to the group A antigen and M protein as well as extracellular products associated with *S. pyogenes*, e.g., streptolysin O (antistreptolysin O test) and DNase B. These tests are used to aid in the diagnosis of patients with sequelae consistent with a past infection with *S. pyogenes*. Sequencing of regions coding for the M protein, a main virulence factor of *S. pyogenes*, can also be used to type strains in epidemiological investigations.

STREPTOCOCCUS AGALACTIAE

Beta-hemolytic streptococci that possess the Lancefield group B antigen are classified as *Streptococcus agalactiae*. While these organisms can cause a variety of human infections, particularly in compromised hosts, they are best known as a leading cause of newborn infections due either to transmission at birth, to maternal colonization of the intestinal and/or genitourinary tract, or to postnatal acquisition.

In an effort to reduce the exposure of newborns, in 2010 the CDC recommended screening of pregnant females at 35 to 37 weeks of gestation by culture for *S. agalactiae*. However, in 2019 the American College of Obstetricians and Gynecologists changed this time frame to 36 to 37 weeks. For detection of colonization of pregnant females, a swab(s) from the distal vagina and anorectum should be collected. Swabs are then used to inoculate blood agar and placed in an enrichment broth containing antibiotics, e.g., colistin (10 μg/ml) or gentamicin (8 μg/ml) and nalidixic acid (15 μg/ml). In the event that the primary plate is negative for *S. agalactiae*, the enrichment broth is subcultured to blood agar after 18 to 24 h of incubation. In general, colonies of *S. agalactiae*, in contrast to *S. pyogenes*, produce a narrow zone of beta-hemolysis on blood-based agar. Alternatively, carrot broth and Granada medium, which turn orange in the presence of *S. agalactiae*, have been used to detect the presence of this organism; however, nonhemolytic strains may not be detected with pigment-dependent differential media. Testing enrichment broth after overnight incubation using nucleic acid amplification techniques (NAAT) has been found to be very sensitive, thus presenting an alternative to culturing on solid agar. NAAT have been used to test females in labor that have not had a screening test performed. However, since the enrichment culture is eliminated in this test, the sensitivity has been shown to be reduced, and this method is therefore not recommended by the CDC.

S. agalactiae can be identified by Lancefield typing using antibodies to detect the group B antigen. In addition, a CAMP (Christie, Atkins, Munch-Petersen) test can be performed to detect a protein, CAMP factor, produced by *S. agalactiae*. To detect the CAMP factor, *S. agalactiae* is streaked at right angles to a strain of *Staphylococcus aureus* that produces a beta-hemolysin that synergistically interacts with the CAMP factor. If *S. agalactiae* is present, hemolysis in the shape of an arrowhead is seen where the two lines of bacterial growth intersect. Disks impregnated with the *Staphylococcus* hemolysin are commercially available that also enable the detection of enhanced hemolysis in the presence of the CAMP factor produced by *S. agalactiae*. Alternatively, *S. agalactiae* can be presumptively identified by its ability to hydrolyze hippurate. Both rapid (2-h) and overnight versions of this test are based on the hydrolysis of hippurate to glycine, which can subsequently be detected with the ninhydrin reagent. Alternatively, MALDI-TOF MS can be used to identify *S. agalactiae*.

STREPTOCOCCUS DYSGALACTIAE SUBSP. *EQUISIMILIS* (LARGE-COLONY BETA-HEMOLYTIC LANCEFIELD GROUPS C AND G)

Human isolates of large-colony beta-hemolytic streptococci that possess either the Lancefield group C or G antigen, and occasionally Lancefield group A and L antigens, are genetically related and have been placed in the same

subspecies, *Streptococcus dysgalactiae* subsp. *equisimilis*. These organisms cause an acute disease spectrum similar to that of *S. pyogenes*; however, they are not generally associated with sequelae, although there are reports of exceptions. *S. dysgalactiae* subsp. *equisimilis* strains are not the only streptococci possessing the Lancefield C and G antigens. Other large-colony strains possessing the group C or G antigen, which may be alpha- or beta-hemolytic or nonhemolytic, are found primarily in animals and can cause zoonoses.

Human isolates are generally identified by typing large-colony beta-hemolytic strains with antibodies to the Lancefield groups. However, small-colony beta-hemolytic organisms can also type as group C or G but are members of the viridans group streptococci, belonging to the anginosus group. Large- and small-colony beta-hemolytic group C and G streptococci can be differentiated from one another by a VP for acetoin or a rapid test to detect β-D-glucuronidase (BGUR), since large-colony isolates are positive by this test but negative by the VP. This enzyme can also be rapidly detected using methylumbelliferyl-β-D-glucuronide-containing MacConkey agar. Unlike with *S. pyogenes* and *S. agalactiae*, to date, identification of *S. dysgalactiae* by MALDI-TOF MS has been problematic, as it has been with identifying members of the viridans group streptococci.

STREPTOCOCCUS PNEUMONIAE

Streptococcus pneumoniae belongs to the *Streptococcus mitis* group of the viridans group streptococci; however, due to their unique phenotypic and clinical manifestations, these species are discussed separately. *S. pneumoniae* is part of the normal respiratory microbiota, and carriage of this organism is common. It is one of the leading causes of community-acquired pneumonia. In addition, it can cause bacteremia, endocarditis, meningitis, sinusitis, and otitis media. There are over 90 serotypes of *S. pneumoniae* based on difference in the polysaccharide capsule. Vaccines incorporating 13 or 23 of these antigens have had an impact in reducing infections caused by *S. pneumoniae*. Traditionally, this organism was universally susceptible to penicillin; however, increasing numbers of strains have developed decreased susceptibility to this first-line antimicrobial agent. A key characteristic of this Gram-positive organism is its lancet-shaped appearance in a Gram stain. Encapsulated strains, which often are more pathogenic due to the antiphagocytic characteristics of the capsule, can be detected by Gram stain when the combination of a proteinaceous background and correct illumination allows a halo (i.e., capsule) around the organism to be visible.

S. pneumoniae grows on blood agar and often needs incubation in 5% CO_2 for optimal growth. Colonies are alpha-hemolytic and can be mucoid in appearance due to a capsule. As the colonies age, they tend to be described as concave and can appear to have a punched-out center. Strains of *S. pneumoniae* have been defined by their capsular antigens, and over 80 serotypes have been identified. Swelling of the capsule in the presence of type-specific antibodies is referred to as a Quellung reaction. Alternatively, strains can be typed using commercially available agglutination tests.

The two tests that are most widely used to identify *S. pneumoniae* are those for bile solubility and optochin susceptibility. Sodium deoxycholate to test for bile solubility, when added to *S. pneumoniae* growing in broth or on solid media, causes lysis of the organisms. Zones of inhibition of ≥14 mm with a 6-mm, 5-μg optochin disk can also be used to distinguish *S. pneumoniae* from other alpha-hemolytic streptococcal organisms. Strains that once were considered *S. pneumoniae* that were not bile soluble or optochin susceptible have recently been included in the species *Streptococcus pseudopneumoniae*, which is also included in the *S. mitis* group. Depending on the database used, misidentification of isolates from the viridans group as *S. pneumoniae* by MALDI-TOF has been reported. However, recent versions of the databases of the two commercially available instruments have shown improvement in correctly identifying *S. pneumoniae*.

Urine and cerebrospinal fluid antigen tests for *S. pneumoniae* are commercially available. These tests have been found to be useful in adult patients that have been treated with antibiotics and in patients with pneumonia who also had a bloodstream infection. Direct NAAT, depending on the application, have been problematic partly due to the inability to distinguish between the normal microbiota and organisms causing respiratory infection. Mixed results have been reported with NAAT when they are used as part of a panel for testing positive blood cultures or cerebrospinal fluid.

VIRIDANS GROUP STREPTOCOCCI

The main species of the viridans group streptococci can be placed in either the bovis, mitis, anginosus, mutans, or salivarius group (Table 2-1). As with other organisms in the genus *Streptococcus*, the viridans group streptococci are normal inhabitants of the mucosal membranes and therefore are commonly found in the gastrointestinal

Table 2-1 Grouping of the viridans group streptococci more commonly associated with human disease

Group	Included species
Bovis	*S. gallolyticus* *S. infantarius* *S. alactolyticus*
Mitis	*S. mitis* *S. pneumoniae* *S. pseudopneumoniae* *S. cristatus* *S. gordonii* *S. oralis* *S. parasanguinis* *S. sanguinis*
Anginosus (milleri)	*S. anginosus* *S. constellatus* *S. intermedius*
Mutans	*S. mutans* *S. criceti* *S. downei* *S. ratti* *S. sobrinus*
Salivarius	*S. salivarius* *S. vestibularis*

and urogenital tracts as well as the oral cavity. Several viridans group species are associated with dental caries and subacute bacterial endocarditis, particularly in patients with damaged or prosthetic heart valves. It is not uncommon to isolate them from polymicrobic abscesses. *Streptococcus intermedius* can be found in deep abscesses, particularly of the brain and liver. Infections with the viridans group are becoming more frequent in neutropenic patients, probably due to the oral mucosal damage from some of the chemotherapeutic agents used.

It has traditionally been difficult to determine the species identity of the viridans group in the clinical laboratory setting. This is in part because they lack characteristic hemolytic reactions in that they can be alpha-hemolytic or nonhemolytic and occasional strains are beta-hemolytic. With the exception of members of the *Streptococcus bovis* group which, along with the genus *Enterococcus*, possess the group D antigen, most viridans group streptococci lack distinct Lancefield antigens. In addition, several nomenclature systems have evolved to describe members within the viridans group. Commercial systems that can identify species in this group are available, and with continued refinement of databases and consolidation of nomenclature, they should prove very useful. Several conventional tests can be used to group and sometimes identify species of the viridans group streptococci. Key tests include the urea hydrolysis test, which is performed on Christensen urea agar incubated at 35°C for 7 days; the VP for acetoin production; the arginine hydrolysis test, which can be done by different methods (depending on the method and the species, results of this test can vary); the esculin hydrolysis test, which can be performed using commercially available slants that are observed for blackening for up to 1 week; fermentation using 1% (wt/vol) carbohydrate in thioglycolate broth containing purple broth base (1.6% [wt/vol]), which is inoculated and incubated anaerobically for 24 h; and the test for hyaluronidase production, which can be detected on agar plates containing 400 μg of hyaluronic acid. The use of fluorogenic substrates has also aided in the differentiation of species of viridans group streptococci; by this method, 4-methylumbelliferyl-linked substrates are degraded, and the by-product can be visualized under UV illumination. Differentiating among the viridans group streptococci by MALDI-TOF MS remains problematic.

S. bovis Group (Group D Streptococci)

Nomenclature of organisms in the bovis group of viridans group streptococci has changed over the years, especially with the knowledge gained from molecular methods; still, as with the other groups within the viridans group, there is often confusion around the grouping and nomenclature of these organisms. Recently, the *S. bovis* group was subdivided based on DNA studies into four clusters. Organisms in cluster I, which are mainly isolated from animals, include strains formerly called *Streptococcus bovis* and *Streptococcus equinus* that are now grouped into a single species, *S. equinus*. Strains in the bovis group causing human infections belong mainly to cluster II. The revised nomenclature for this group includes *Streptococcus gallolyticus*, which comprises three subspecies, *Streptococcus gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *pasteurianus*, and *S. gallolyticus* subsp. *macedonicus*. Importantly, there is a strong association of isolation of *S. gallolyticus* from blood cultures with colorectal cancer. In addition, *S. gallolyticus* can cause bacteremia, endocarditis, and meningitis. Also clinically important are *Streptococcus infantarius* subsp. *infantarius* and *Streptococcus infantarius* subsp. *coli* in cluster III. Cluster IV includes *Streptococcus alactolyticus*, which also has been reported to be isolated from human infections, although less commonly than other members in this group.

Members of the *S. bovis* group are alpha-hemolytic or nonhemolytic on blood agar, which is commonly used to isolate these organisms. They can be differentiated from other alpha-hemolytic and nonhemolytic