**GRAM-POSITIVE BACTERIAL PATHOGENS**

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**Introduction**

Gram-positive pathogens possess various advantageous characteristics and mechanisms that allow them to adhere to, colonize, and attack host cells. They can cause a range of diseases, from food poisoning and sore throat to severe respiratory infections and nervous system-related illnesses. Among these, many are highly fatal acute infections, with mortality rates reaching up to 80% in some cases.

This chapter will introduce to you the morphological, biochemical and molecular distinct properties, the culture conditions and frequently used culture media, as well as the diseases, treatments, and prevalence of most important gram-positive pathogens in the present clinical settings, including *Bacillus cereus, Clostridium difficile, Corynebacterium jeikeium, Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae,* and *Streptococcus pyogenes.*

**BACILLUS CEREUS**

**Morphological characteristics:** *Bacillus cereus*, a Gram-positive rod- shape bacterium, displays a distinctive purple or blue hue owing to its robust peptidoglycan layer, which retains the stain during Gram staining. The name “cereus”, which means waxy in Latin, comes from its large, feathery, and dull gray colonies appearance on blood agar plates. Its colonies are also granular, spreading with opaque, rough matted surfaces and irregular perimeters. Typically arranged in chains or pairs, although occasionally as solitary bacilli, *B. cereus* is encapsulated with a polysaccharide capsule which protects it from the environment and from being devoured by the host immune system. Under unfavorable conditions such as food scarcity, it can generate spores which can withstand high temperature for long periods of time. This pathogen has many long flagella which signifies its high motility in both swimming and swarming.

**Figure 1:** *Gram-stained Bacillus cereus photomicrograph showcases highly motile flagella. Leifson flagella stain method was used to achieve the optimal observation of the flagella.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Dr. William A. Clark, 1977*

**Figure 2***: Gray, dull and featherly colonies of Bacillus cereus on sheep blood agar medium (SBA). The name “cereus", which means wax-like, is based on the appearance of colonies.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC)/ Courtesy of Larry Stauffer, Oregon State Public Health Laboratory, 2002.*

**Phenotypic and biochemical properties:** *B. cereus* is a facultative anaerobe, meaning it is able to thrive in both oxygen-rich and oxygen-poor environments. Remarkably, it exhibits catalase positivity, indicating its ability to break down hydrogen peroxide into water and oxygen. Moreover, it is beta-hemolytic which can completely lyse red blood cells on blood agar plates. Notably, *B. cereus* produces a heat-stable toxin (enterotoxin 1) that causes food poisoning in reheated food, especially in reheated rice, particularly known as the "fried rice syndrome".

**Culture condition and media:** *B. cereus* is selectively cultured in Mannitol yolk polymyxin B agar (MYPA) and polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA). These media selectivity depends on the capacity of *B. cereus* to ferment mannitol or hydrolyze egg yolk, respectively (Mayer, 2015). Another option is Brilliance Bacillus cereus Agar (BBC) which contains the antibiotic trimethoprim which improves selectivity (Mayer, 2015). BBC shows effectiveness in inhibiting background microflora in unpasteurized food samples and is considered to be a better option among MYPA, PEMBA and BBC (Chon et al., 2014).

**Molecular identification:** Polymerase chain reaction (PCR) is used for the detection of B. cereus by amplification and sequencing of target genes such as 16S rRNA and virulence factor genes including non-haemolytic exotoxin genes *nheA/nheB/nheC*, hemolysin genes *hblC/hblD/hblA*, cereulide synthetase gene *cesB*, and cytotoxin K gene *cytK* (Liu et al., 2020). Moreover, MALDI-TOF mass spectrometry can be reliably identify *B. cereus* and distinguish it from closely related species like *B. thuringiensis* based on the intensity profiles of small molecule biomarkers in the low-mass range (m/z 500–3,000) (Ha et al., 2019).

**Virulence factors:** The virulence factors of *B. cereus* include a range of toxins and substances that contribute to its pathogenicity and ability to cause food poisoning and infections. One key virulence is Hemolysin BL (Hbl) - a protein known for its hemolytic, cytotoxic, dermonecrotic, and capillary permeability properties. Nonhemolytic Enterotoxins (Nhe) consist of enterotoxin 1 and 2 which are the major causation of foodborne infections in humans (Lindbäck et al., 2004). Enterotoxin 1 is heat stable; hence, upon food reheating, *B. cereus* produces this toxin and causes nausea and vomiting within 30 minutes to 6 hours post-ingestion. Enterotoxin 1 is the reason *B. cereus* is famously known for inducing “fried rice syndrome", a syndrome where food poisoning is caused by reheating rice. Meanwhile, enterotoxin 2, produced while the pathogen is already within the intestine, triggers diarrhea and abdominal pain 6 to 15 hours after consumption. Other toxins of *B. cereus* include Cytotoxin K (CytK) - a cytotoxic protein that affects capillary permeability and cytotoxicity in the host; Enterotoxin FM (EntFM) which is linked to cytotoxicity and capillary permeability, and Emetic Toxin (Cereulide) which is responsible for emetic-type food poisoning (Owusu-Kwarteng et al., 2017).

**Diseases and treatments:** *B. cereus* is a foodborne pathogen that can produce two enterotoxins, causing two types of gastrointestinal illness: ​​the emetic (vomiting) syndrome associated with enterotoxin 1, and the diarrhoeal syndrome associated with enterotoxin 2. Aside from food poisoning, *B. cereus* is also involved in infections of eyes, respiratory tract, and wounds (McDowell et al., 2023).

**Prevalence:** *B. cereus* prevalence is higher in developing countries compared to developed countries where Australia has the lowest rate; however, there is an exception of America which possesses the highest rate of prevalence. The overall prevalence of *B. cereus* is 23.746% (CI 95%). Moreover, prevalence of *B. cereus* depends on the types of food as well as the detection method. A study showed that this pathogen was found the highest in cereals and beans, the second highest in vegetables and dairy products. Molecular tests such as PCR report twice the prevalence compared to other morphological/biochemical methods (Rahnama et al., 2023).

**CLOSTRIDIUM DIFFICILE**

**Morphological characteristics:** *Clostridium difficile (C. difficile*) is a Gram-positive, rod-shape, anaerobic and spore-forming bacterium. The rods are usually straight or slightly curved. Individual cells of *C. difficile* can vary in size, but generally around 1.0 to 1.5 micrometers in width and 4.0 to 6.0 µm in length (Markovska et al., 2023).

***Figure 3:*** *This photomicrograph reveals the presence of numerous Clostridium difficile bacteria in an impression blood agar impression smear, which had been incubated for 72 hours, in an anaerobic environment.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Dr. Gilda Jones, 1980.*

**Phenotypic and biochemical properties:** Under Gram staining, *C. difficile* cells are Gram-positive and demonstrate optimal development on blood agar at human body temperatures under anaerobic conditions. It is catalase and superoxide dismutase negative and produces 2 types of toxins: enterotoxin A and cytotoxin B, which disrupts cytoskeleton signal transductions in the host.

**Culture conditions:** *C. difficile* grows rapidly at 37oC in the absence of oxygen. CCFA medium which contains Cycloserine, Cefoxitin, Fructose, and Egg yolk is the selective and differential agar medium used to facilitate the isolation of *C. difficile* from fecal samples. CCFA was found to be the most selective and sensitive medium to recover *C. difficile* (George et al., 1979).

**Identification methods:** Using PCR method to detect *C. difficile* toxin gene. These tests are rapid and very sensitive methods to confirm the presence of *C. difficile* toxin gene. False positives with this method can be minimized by only testing those people who are symptomatic and likely to have *C. difficile* infection (CDI). Other tests that may sometimes be performed to detect *C. difficile* include: gastrointestinal pathogen panels, cell cytotoxicity assay, toxigenic stool culture. Cytotoxicity assay (also known as tissue culture assay) is the gold standard for the diagnosis of *C. difficile*. The presence of *C. difficile* toxin is confirmed by the anti-toxin antibodies' neutralization of the cytotoxic effect. It is a sensitive method to detect toxins, however the test results take 24 to 48 hours which hinder its application in clinical laboratories.

**Virulence factors and antibiotic resistance genes/proteins:** *C. difficile* produces many exotoxins that serve as its primary pathogenic agents. The two primary toxins are TcdA, which has a size of 308 kDa, and TcdB, which has a size of 270 kDa. These toxins exhibit both enterotoxic and cytotoxic effects by functioning as glycosyltransferases that deactivate human Rho GTPases. This deactivation results in elevated levels of proinflammatory cytokines (IL-1, TNFα, IL-8), infiltration of leukocytes, inflammation, secretion of fluids, depolymerization of actin filaments, disruption of tight junctions, apoptosis of cells, and damage to the gut epithelium. Pseudomembranous colitis, characterized by thick yellow fibrous exudates, can occur in severe instances. Although TcdA and TcdB share 47% of their structural characteristics, they have separate processes and interact with different receptors. Based on tests conducted on human tissue, Toxin B is considered more potent, and monoclonal antibodies against TcdB are used in treatment, underscoring its significance (Markovska et al., 2023).

*C. difficile* also produces other enzymes, such as collagenase, chondroitin sulfatase, and hyaluronidase, which disrupt tight junctions, leading to fluid loss. Additional elements that contribute to the ability of a pathogen to cause disease include surface proteins, specifically the S layer, and the production of durable spores. Spores have a significant impact on the recurrence and long-term survival in the environment. Biofilm generation is a factor in the pathogenicity of *C. difficile*, although its mechanisms are not completely known. It contributes to the modulation of the immune response and the recurrence of the infection (Markovska et al., 2023).

**Diseases and treatment:** *C. difficile* is spread in the form of dormant spores by the route of fecal-oral transmission. Once within the gastrointestinal system, these spores develop into active cells that can produce several toxins, leading to the development of severe illness and colitis (Edwards et al., 2013). Symptoms of *C. difficile* infection include diarrhea (at least three loose bowel movements a day), a high temperature, dehydration, abdominal pain that can be severe, loss of appetite, and nausea.

*C. difficile* treatment involves careful use of antibiotics, which are both a treatment and a risk factor for infections. Standard therapy includes metronidazole and vancomycin, with vancomycin being more effective. Fidaxomicin is crucial for treating CDIs and preventing recurrences. For an initial infection, only vancomycin or fidaxomicin are recommended, while metronidazole may be used in combination with vancomycin.

If CDI develops during antibiotic treatment, the antibiotic should be stopped or switched to lower-risk options like macrolides, aminoglycosides, sulphonamides, vancomycin, or tetracyclines. Bezlotoxumab, a monoclonal antibody for preventing recurrent CDI that the FDA approved in 2016, is one of the new treatments being considered. Fecal microbiota transplantation (FMT) has shown a 75–90% success rate in preventing recurrences (Cheng & Fischer, 2023). Other emerging treatments involve toxin blockers like calcium aluminosilicate, human serum albumin, aspirin, human alpha-defensins, and ambroxol.

**Prevalence and prevention:** Most cases of pseudomembranous colitis and 25-30% of cases of antibiotic-associated diarrhea are caused by *C. difficile* (Biswas et al., 2023). People most at risk for CDI are the elderly, people in healthcare settings, and those who have received antibiotic treatment. In 2011, CDI killed nearly 500,000 people in the USA and about 29,000 people (Sandhu & McBride, 2018). CDI is the most common healthcare-related infection in the USA, leading to increased healthcare costs of $4.8 billion.

Currently, there is no commercially available vaccination for *C. difficile*. Regrettably, clinical experiments with *C. difficile* toxin-based antigens did not demonstrate sufficiently high efficacy, as they failed to prevent the colonization and spread of the pathogen among patients (Razim et al., 2023). CDI can be effectively decreased through a combination of prudent antimicrobial prescribing and infection control measures such as environmental decontamination, hand hygiene, isolation, and the use of personal protective equipment (Gouliouris et al., 2011).

**CORYNEBACTERIUM JEIKEIUM**

**Morphological characteristics:** *Corynebacterium jeikeium* (*C. jeikeium*) is gram-positive, catalase-positive, non-spore-forming, aerobic, non-motile, rod-shaped bacteria. Individual cells vary in size but are generally around 0.5 to 1.5 micrometers in width and 2.0 to 6.0 µm in length. Corynebacterium cells may occur singly, in pairs, or V shaped or palisade arrangements. Some species may also form characteristic Chinese letter-shaped arrangements. On blood agar, they form small greyish colonies with a granular appearance, mostly translucent, but with opaque centers, convex, with continuous borders.

**Phenotypic and biochemical properties:** The bacterium is aerobic or facultatively anaerobic and exhibits a fermentative metabolism (carbohydrates to lactic acid) under certain conditions. They are fastidious organisms, growing slowly even on enriched medium. *C. jeikeium* is non-hemolytic, urease-negative, and catalase positive.

**Culture conditions:** Tellurite Blood Agar is a selective medium used for the isolation and cultivation of Corynebacterium species. In nutritional requirements, all need biotin to grow. The bacteria also grow in Loeffler's medium, blood agar, and trypticase soy agar (TSA). Their optimum growth temperature is 37° C.

**Identification methods:** In clinical practice settings, different biochemical test systems have been developed to identify Corynebacterium species. Rapid 4-hour tests are performed for urease, pyrazinamidase, catalase, and nitrate reduction. *C. jeikeium* is nitrate-negative, urease-negative, catalase-positive and pyrazinamidase-positive. For further identification, typing methods, whole genome sequencing, 16S rRNA gene (rDNA) sequence analysis can be used.

**Virulence factors:** Predicted virulence factors of *C. jeikeium* are degrading enzymes involved in exogenous fatty acids metabolism via damaging the host tissue (Tauch et al., 2005).

**Diseases and treatment:** *C. jeikeium*, a resident of human skin, is often associated with multidrug resistant nosocomial infections.Prolonged hospital stays, treatment with broad-spectrum antibiotics, and impaired skin integrity are risk factors for infection with *C. jeikeium*. Other infections attributed to *C. jeikeium* include skin and wound infections, catheter-related infections, enteritis, meningitis, osteomyelitis, peritonitis, pneumonia, prosthetic joint infections, pyelonephritis, and liver abscess in a patient with acquired immunodeficiency syndrome (AIDS).

*C. jeikeium* endocarditis is extremely difficult to treat as it is characteristically resistant to penicillin, cephalosporins, and aminoglycosides, and sensitivity to quinolones, macrolides, tetracyclines and rifampin is variable. However, it remains susceptible to vancomycin, the most active antibiotic agent and the drug of choice in *C. jeikeium* infections (Rezaei Bookani et al., 2017).

**Prevalence and prevention:** Proportions of true bacteremia cases caused by *C. jeikeium* was 71% which was significantly higher than those caused by other species of Corynebacterium (9%) (Yamamuro et al., 2021). *C. jeikeium* has a high infection-related mortality of approximately 30%; as it is typically multidrug-resistant and can form biofilms (Dowling & Koen, 2020). Preventing nosocomial transmission of *C. jeikeium* relies on thorough handwashing and aseptic procedures.

**ENTEROCOCCUS FAECALIS**

**Morphological characteristics:** *Enterococcus faecalis (E. faecalis)* is Gram-positive, nonmotile, non-spore-forming bacterium in nature and is found in the upper respiratory tract, intestine, and mouth of healthy people. Colony morphology is small, gray, and γ-hemolytic.

***Figure 4:*** *This illustration depicts a three-dimensional (3D) computer-generated image of a cluster of paired, or diplococcal, vancomycin-resistant Enterococcus (VRE) bacteria.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Dan Higgins, 2013.*

**Phenotypic and biochemical properties:** *E. faecalis* ferments glucose without gas production and does not produce a catalase reaction with hydrogen peroxide. It catabolizes a variety of energy sources, including glycerol, lactate, malate, citrate, arginine, agmatine, and many keto acids. It survives in very harsh environments, including extremely alkaline pH (9.6) and salt concentrations. They exhibit resistance to bile salts, detergents, heavy metals, ethanol, azide, and desiccation. They can survive in high salt concentrations at 10-40°C and high pH at temperatures of 60 °C for 30 minutes (Morandi et al., 2005)

**Culture conditions:** *E. faecalis* can grow on blood agar, bile esculin agar, brain heart infusion agar/broth, and tryptic soy agar. Enterococcosel agar is a selective medium containing bile salts and sodium azide for isolating and differentiating *Enterococcus* species from other bacteria.

**Identification methods:** In order to confirm the identity of *E. faecalis*, we utilized a PCR assay that detects internal segments of the ddl gene, which encodes D-alanine-D-alanine ligase. This gene is used as a diagnostic marker that distinguishes between the two primary clinically significant species, *E. faecalis* and *E. faecium*. (Dutka-Malen et al., 1995).

**Virulence factors and antibiotic resistance genes/proteins:** This bacterium has developed multi-drug antibiotic resistance and uses colonization and virulence factors to form biofilms. *Enterococcal* surface protein aggregates bacteria, while aggregation substances cytosolin and gelantinase facilitate the transfer of genetic material between cells. These factors help regulate adherence and inhibit competitive bacteria, allowing the bacteria to bind to target cells. Biofilms are responsible for around 65% of bacterial infections and can exhibit up to 1000 times more resistance to antibiotics compared to individual planktonic cells (M.-A. Kim et al., 2020).

**Diseases and treatment:** *Enterococci* have the potential to cause urinary tract infections, peritonitis, sepsis, or endocarditis. They frequently form part of a diverse collection of plants*. E. faecalis* is recognized as a causative agent of infective endocarditis, which is an infection of the heart valves. This infection primarily affects individuals with previous heart issues or those who have undergone invasive medical operations.

*Enterococcus* infections, including Vancomycin-resistant *Enterococci* (VRE) infections, cause a range of different symptoms depending on the location of the infection. This includes infections of the bloodstream, urinary tract infections, and wound infections associated with catheters or surgery. Linezolid or daptomycin are used for treating VRE infections. Alternatively, assuming it is non-VRE, we have the option to provide either ampicillin or vancomycin.

**Prevalence and prevention:** *Enterococci* are widely distributed in nature. They are found in such diverse habitats as the gastrointestinal tract, oral cavity, and upper genital tract of man and other mammals, and also in birds, reptiles, insects, plants, soil, and water (Morandi et al., 2005). *Enterococci* have become recognized as serious nosocomial pathogens causing bacteremia, endocarditis, and infections of the urinary tract. *E. faecalis* is responsible for about 80–90% of all *enterococcal* infections and *E. faecium* accounts for most others (Sreeja et al., 2012).

Preventing infections caused by *E. faecalis*, particularly in healthcare settings, involves several strategies: infection control practices (hand hygiene, use of personal protective equipment, environmental cleaning), antibiotic stewardship (limiting the use of broad-spectrum antibiotics and following guidelines for appropriate antibiotic), aseptic insertion and maintenance (following strict aseptic techniques during insertion and maintenance of catheters and other invasive devices to prevent infections).

**LISTERIA MONOCYTOGENES**

**Morphological characteristics:** *Listeria monocytogenes* is a Gram-positive bacterium which appears purple or blue under microscopic observation. It is rod-shaped and cannot form spores. Notably, *L. monocytogenes* exhibits motility, showcasing a unique tumbling motility, motile at 22°C and non-motile at 35-37°C. At the optimal temperature of 22°C, its means of motility is flagella, however, at body temperature of around 37°C where flagella is no longer useful, it utilizes special filaments called actin rockets to move within host cells. Actin filaments form part of the cytoskeletal framework supporting various cellular processes, and *Listeria* manipulates these filaments to move intracellularly and intercellularly, facilitating the rapid spread of infection.

***Figure 5:*** *Listeria monocytogenes isolated from a neonatal listeriosis patient's lung. Levaditi silver impregnation method was used to histochemically process the specimen.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Dr. Heinz Seeliger, 1965.*

***Figure 6:*** *Flagella of Listeria monocytogenes was observed under transmission electron microscopic (TEM) at very high magnification of 41,250X.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Elizabeth White, 2002.*

**Phenotypic and biochemical properties:** *L. monocytogenes* is catalase-positive, possessing the enzyme catalase, which aids in the breakdown of hydrogen peroxide into water and oxygen, enabling the bacterium to withstand oxidative stress within host environments. However, it is oxidase-negative, lacking the enzyme cytochrome c oxidase in its electron transport chain. Furthermore, *L. monocytogenes* displays beta-hemolytic activity, causing complete lysis of red blood cells on blood agar, leading to a clear zone around the colonies. Additionally, it is facultatively anaerobic, capable of thriving in both aerobic and anaerobic conditions, facilitating its adaptability to diverse environments, which contributes to its ability to infect a wide range of hosts and survive in various niches. These biochemical attributes contribute significantly to the pathogenicity and survival strategies of *L. monocytogenes*.

**Culture condition and media:** *L. monocytogenes* is an auxotrophic bacterium that requires seven amino acids (leucine, isoleucine, valine, methionine, arginine, cysteine, and glutamine) and four extra vitamins (thiamine, biotin, riboflavin, and thioctic acid) in order to grow (Premaratne et al., 1991). Brain Heart Infusion (BHI) is a non-selective medium frequently used for cultivating *Listeria* species since it can offer these growth elements (Jones & D'Orazio, 2013). In addition, commonly recommended selective media for the cultivation of *L. monocytogenes* include PALCAM agar and Oxford agar. PALCAM agar consists of Columbia Blood Agar and its selectivity is achieved by the addition of polymyxin B, acriflavine, ceftazidime, and esculin. After 24-48 hours of incubation at 37°C, *L. monocytogenes* colonies on PALCAM agar appear gray-green in color with a black sunken center and a black halo (Law et al., 2015). Oxford medium consists of Columbia Blood Agar and its selectivity is achieved by addition of lithium chloride, acriflavine, colistin sulfate, cycloheximide, cefotetan, and fosfomycin. After 24 hours of incubation at 37°C, *L. monocytogenes* colonies on Oxford agar appear olive-green with a black halo, and after 48 hours they turn darker with a black sunken center and black zones (Law et al., 2015).

**Molecular identification:** PCR is a highly sensitive and specific molecular technique used for the rapid detection and identification of *L. monocytogenes*. PCR can target specific genes unique to the pathogen including internalin genes *inlA/inlB*, listeriolysin O precursor gene *hly*, phospholipase gene *plcA/plcB*, actin-assembly inducing protein precursor gene *actA* (Joseph et al., 2006). Along with PCR, enzyme-linked immunosorbent assay (ELISA) and DNA hybridization has also been used and showed equal sensitivity. In recent times, aside from DNA-based tests, RNA-targeting molecular techniques like real-time quantitative polymerase chain reaction (RT-qPCR) and nucleic acid-based sequence amplification (NASBA) have been created. These tests can be used for quantitative analysis in addition to providing a measure of cell viability (Gasanov et al., 2005).

**Virulence factors:** One major characteristic of *L. monocytogenes* is the actin filaments, which are commonly referred to as “rocket tails” and especially helpful for propelling within host cell's cytoplasm in low temperature. Surface protein ActA is the virulence factor essential for inducing actin polymerization and forming actin comet tails in the pathogen (Coelho et al., 2019). Other surface-exposed proteins are internalin A and B (InlA/InlB), with InlA primarily mediating entry into epithelial cells and InlB facilitating entry into a broader range of cell types. These internalins also contribute to the traversal of anatomical barriers, such as the intestinal, blood-brain, and placental barriers, enabling the bacterium to cause systemic infections (Ireton et al., 2021). Another major virulence factor of *L. monocytogenes* is Listeriolysin O (LLO) - a pore forming toxin. LLO can break phagosome membranes, enable the pathogen to enter the cytosol, proliferate and minimize harm to its replicative environment within the host cell's cytosol. An additional virulence aiding in the activity of Listeriolysin O is phospholipase PlcB, which regulates the quantity and accessibility of cholesterol in the lipid membrane (Petrišič et al., 2021).

**Diseases and treatments:** *L. monocytogenes* can cause a series of diseases known as Listeriosis, a foodborne disease acquired by ingestion of unpasteurized dairy products and cold deli meats. *L. monocytogenes* grows very well at cold temperatures, therefore storage of contaminated food in the refrigerator can increase the risk of infection. And this paradoxical growth in the cold is called “cold enhancement”. Listeriosis has a high mortality rate of 20-30% and affects most severely on pregnant women, adults above 65, infants, and immunocompromised patients. Infection in immunocompromised people can cause gastroenteritis with symptoms of watery diarrhea, fever, and abdominal cramps. In pregnancy, *L. monocytogenes* infection can cause abortion and premature delivery. And newborns infected at the time of delivery as well as immunocompromised individuals can develop meningitis, which usually presents with fever, neck stiffness, headache, altered mental status, and other classic signs of meningitis (Jamshidi et al., 2009). Antibiotics used to treat Listeriosis are ampicillin and in some cases combined with gentamicin (Temple 7 Nahata, 2000).

**Prevalence:** Prevalence of *L. monocytogenes* infections and listeriosis in most industrialized countries has decreased over the past several decades due to improved sanitary methods. However, pregnant women still have a tendency of infection. The prevalence was found to be 3.66% among women with spontaneous abortion, 1.83% among women with normal delivery, and 3% among fertile women (Ahmadi et al., 2022). Furthermore, in South-East Asia, the pathogen was identified to exist 16% in food, animal, and environmental sources (Jibo et al., 2022). Interestingly, one study found that in a tropical country such as Ecuador, prevalence of *L. monocytogenes* doubled in the dry season (22.2%) compared to the rainy season (10.4%). The study also reported that this pathogen is detected in 22.5% in mortadella, 19% in hamburger meat, 15% in bacon, 14.5% in chorizo paisa and 10.5% in salami (Meza-Bone et al., 2023). These numbers are comparatively higher than those recorded from a temperate country (deli meat at 2.9%, soft cheese at 2.4%, packaged salad at 2.0%) (Churchill et al., 2019).

**STAPHYLOCOCCUS AUREUS**

**Morphological characteristics:** *Staphylococcus aureus (S. aureus)* is a Gram-positive cocci-shaped bacterium. *S. aureus* is a non-spore-forming, nonmotile, and facultative anaerobes (not requiring oxygen). They are often in clusters resembling a bunch of grapes when observed under a light microscope after Gram staining. *S. aureus* forms fairly large yellow or white colonies on nutrient-rich agar media. Carotenoids produced by the organism impart the yellow color of the colonies. The organism is often hemolytic in blood agar because of the production of four types of hemolysins (alpha, beta, gamma, and delta). The size of individual cells can vary, but they are typically around 0.5 to 1.0 micrometers (µm) in diameter.

***Figure 7:*** *This digitally colorized, scanning electron microscopic (SEM) image, depicts four magenta-colored, spherical, methicillin-resistant, Staphylococcus aureus (MRSA) bacteria, that were in the process of being phagocytized by a blue-colored human white blood cell (WBC)*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), National Institute of Allergy and Infectious Diseases (NIAID), 2011.*

**Phenotypic and biochemical properties:** Some important biochemical features of *S. aureus* help to identify and comprehend its pathogenicity. These properties include catalase production, which breaks down hydrogen peroxide into water and oxygen, and coagulase production, which converts fibrinogen to fibrin, leading to clot formation (Cheung et al., 2021). Hemolysin production is one of the important features of *S. aureus*. This enzyme lyses red blood cells, resulting in a clear zone around bacterial colonies on blood agar plates. Mannitol fermentation is another key feature, with *S. aureus* fermenting mannitol salt agar, resulting in acid production that turns the agar yellow. These biochemical characteristics contribute to *S. aureus* virulence and are also used for identification.

**Culture conditions:** *S. aureus* produces the yellow pigment staphyloxanthin and characteristic gold-colored colonies are formed on all rich media such as tryptic soy agar (TSA) at a temperature of 37°C, brain heart infusion (BHI) agar, and Luria Bertani (LB) agar. *S. aureus* develops black colonies surrounded by a zone of lipid precipitation when grown on Baird-Parker agar with egg yolk tellurite. This lipid precipitation is induced by the release of glycerol-ester hydrolases (lipases) (Rosenstein & Götz, 2000). The presence of tellurite and lithium chloride in Baird-Parker agar inhibits the development of the majority of bacteria, whereas pyruvate and glycine selectively stimulate the growth of *S. aureus*. Tryptic soy broth (TSB) and BHI are the optimal media for cultivating *Staphylococci* cultures. Cultures are incubated at a temperature of 37°C with aeration.

**Identification methods:** Initially, a Gram stain is performed as a preliminary step to provide guidance. This stain should reveal the presence of characteristic Gram-positive bacteria, specifically cocci arranged in clusters. Next, the isolate is grown on mannitol salt agar, a specialized medium containing 7.5% NaCl that promotes the growth of *S. aureus*. This growth leads to the formation of yellow colonies due to the fermentation of mannitol and the consequent decrease in the pH of the medium. In addition, to differentiate between species, various tests are conducted including catalase (positive for all Staphylococcus species), coagulase (positive for *S. aureus*, indicated by fibrin clot formation), DNAse (indicated by a zone of clearance on DNase agar), lipase (indicated by a yellow color and rancid odor), and phosphatase (indicated by a pink color). The coagulase test is used to determine whether an infection is caused by *S. aureus.*

**Virulence factors and antibiotic resistance genes/proteins:** *S. aureus* is a pathogen that produces various enzymes and toxins that enhance its pathogenicity and adaptability. These enzymes include coagulase, which converts fibrinogen to fibrin, hyaluronidase, which breaks down hyaluronic acid, deoxyribonuclease, which degrades DNA, lipase, staphylokinase, and beta-lactamase, which promote the spread of infections. Additionally, S. aureus secretes several exotoxins, which are associated with specific diseases. Superantigens, such as 25 identified staphylococcal enterotoxins (SEA - SEZ) and toxic shock syndrome toxin (TSST-1), can cause toxic shock syndrome (TSS), characterized by fever, rash, low blood pressure, shock, organ failure, and skin peeling. Some strains also produce enterotoxins that cause gastroenteritis, nausea, vomiting, diarrhea, and abdominal pain. Exfoliative toxins are responsible for staphylococcal scalded skin syndrome (SSSS), primarily affecting infants and young children. *S. aureus* also produces other toxins that act on cell membranes, such as alpha, beta, and delta toxins, and bicomponent toxins like Panton-Valentine leukocidin (PVL), which is associated with severe necrotizing pneumonia in children. These enzymes and toxins significantly contribute to the virulence and adaptability of S. aureus, making it a formidable pathogen.

*S. aureus* biofilms play a crucial role in the development of diseases, as they can enhance resistance to antibiotics and evade the immune system. The biofilm formed by *S. aureus* exhibits significant resistance to both antibiotic treatments and the immunological response of the host.

***Figure 8:*** *This image depicts a Petri dish culture plate containing an unidentified growth medium, which had been inoculated with the facultative anaerobic bacterium, Staphylococcus aureus. This dish was created in order to perform an antimicrobial sensitivity test, and was incubated in an anaerobic environment.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Don Stalons, 1972.*

**Diseases and treatment:** The natural habitats of *S. aureus* are healthy people's nose, throat, hair, skin, and mucous membranes. Most infections caused by *S. aureus* are skin and soft tissue infections such as abscesses or cellulitis. S. aureus can also induce severe infections, including pneumonia (lung infection) and bacteremia (bloodstream infection). Malaise, fever, shivers, and difficulty breathing are some of the symptoms of these infections.

Infections involving *S. aureus* are treated with antibiotics. Penicillin is the preferred treatment for *S. aureus* infection in susceptible strains. However, in the majority of countries, penicillin resistance is exceedingly prevalent (>90%). The initial treatment is typically a penicillinase-resistant β-lactam antibiotic (e.g., oxacillin or flucloxacillin, which share the same mechanism of action as penicillin) or vancomycin, based on the local resistance patterns. Methicillin-resistant Staphylococcus aureus (MRSA) is one of several strains of *S. aureus* that have developed resistance to the majority of β-lactam antibiotics. Vancomycin, a glycopeptide antibiotic, is often used to treat MRSA as a result.

**Figure 9:** *The lesions on the volar surface of this patient’s left forearm proved to be Streptococcal impetigo, a dermatologic condition quite often caused by Staphylococcus aureus bacteria*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC),Dr. Herman Miranda, Univ.of Trujello, Peru; A. Chambers, 1964.*

**Prevalence and prevention:** In 2019, *S. aureus* was identified as the second most prevalent pathogen causing deaths associated with antimicrobial resistance (Zhang et al., 2023). Developing a vaccine against *S.aureus* has been unsuccessful for over a decade. StaphVAX (Nabi Biopharmaceuticals, Rockville, MD) and V710 (Merck, Kenilworth, NJ) are notable examples. There are numerous reasons for these failures, but one of the most significant has been the challenge of identifying correlates of protective immunity to *S.aureus.*

**STAPHYLOCOCCUS EPIDERMIDIS**

**Morphological characteristics:** *Staphylococcus epidermidis (S. epidermidis)* is a Gram-positive bacterium belonging to the genus Staphylococcus*.* It is a coagulase-negative, catalase-positive, and facultative anaerobic bacteria that forms clusters. *S. epidermidis* cells are in cocci shape which is around 0.5 to 1.5 micrometers in diameter. It is frequently isolated from human epithelium, especially from axillae, nares, and the head.

**Figure 10:** *Two Staphylococcus epidermidis bacteria observed by scanning electron microscopic (SEM) and digitally colored.*

*Source: Public Health Image Library, Center for Disease Control and Prevention, Janice Haney Carr.*

**Phenotypic and biochemical properties:** *S. epidermidis* is a Gram-positive bacterium. It is oxidase-negative and catalase-positive. The bacterium shows negative results for the Methyl Red test and Indole test. In contrast, it shows positive for hydrogen sulfide (H₂S) production, urease, and nitrate reductase. Moreover, it is positive for the Voges-Proskauer test and β-galactosidase activity (Paul et al., 2021). These biochemical properties help distinguish *S. epidermidis* from other *Staphylococcus* species and other genera.

**Culture conditions:** Staphylococci are generally cultured in blood, tryptic soy, or heart infusion agar. The culture can also be introduced onto mannitol salt agar containing 7.5% sodium chloride, as *S. epidermidis* can tolerate high salt concentrations.

**Identification methods:** *S. epidermidis* is usually detected by colonies on selective media, light microscopy, catalase, and slide coagulase testing. Zobell agar is useful for the isolation of *S. epidermidis* from marine organisms (Paul et al., 2021). On the Baird-Parker agar with egg yolk supplement, colonies appear small and black. Real-time polymerase chain reaction is also being used to rapidly detect *S. epidermidis* (Kim et al., 2021). If available, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) can be used to distinguish different CoNS. The final diagnosis relies on the combination of clinical symptoms and paraclinical studies. The selected tests depend not only on the suspected infection type but also on local guidelines.

**Virulence factors and antibiotic resistance genes/proteins:** Biofilm formation is the primary virulence factor of *S. epidermidis* as it produces an extracellular material called polysaccharide intercellular adhesin (PIA), which allows bacteria to bind to the existing biofilm, creating a multilayer biofilm. This decreases the metabolic activity of bacteria within the biofilm, making it difficult for antibiotics to effectively clear the infection (Otto, 2009). *S. epidermidis* strains are often resistant to antibiotics, including rifamycin, fluoroquinolones, gentamicin, tetracycline, clindamycin, and sulfonamides. Methicillin resistance is particularly widespread, with 75-90% of hospital isolates resistance to methicillin (Otto, 2009). Methicillin-Resistant *Staphylococcus epidermidis* (MRSE) strains harbor the mecA gene, conferring resistance to methicillin and other beta-lactam antibiotics. This resistance complicates treatment options and enhances the persistence of infections.

**Diseases and treatment:** *S. epidermidis* is a prevalent symbiotic organism found on the skin and mucous membranes of humans and other mammals. Typically, *S. epidermidis* poses no threat to individuals in good health. It is a disease with low virulence that can infect patients with weakened immune systems or those with implants. Moreover, *S. epidermidis* can form biofilm on any abiotic surface.

**Prevalence and prevention**: *S. epidermidis* is responsible for 30 to 43% of infections in artificial joints. It also causes about 22% of bloodstream infections in intensive care units, almost 13% of infections in prosthetic valves, and most cases of sepsis in neonates. These infections increase mortality, prolonged hospitalization, and additional surgeries (Otto, 2009). Vaccination and decolonization are not suitable for *S. epidermidis*. It is widely accepted that prevention is the most effective approach to managing S. epidermidis infections (Skovdal et al., 2022). This involves sterilizing medical equipment and the body parts of patients as well as doctors who may come into contact with indwelling medical devices during surgery.

**STREPTOCOCCUS PNEUMONIAE**

**Morphological characteristics:** *Streptococcus pneumoniae* is a gram-positive, lancet-shaped diplococci, typically occurring in pairs of two cocci under microscopic examination. This pathogen is encapsulated in polysaccharides, which protects against phagocytosis, facilitating colonization and invasion of host tissues. Additionally, *S. pneumoniae* expresses surface proteins like pneumococcal surface protein A (PspA) and pneumolysin, pivotal in colonization and pathogenesis. Furthermore, it utilizes various adhesins to adhere to host cells and mucosal surfaces, facilitating colonization and the establishment of infections. These features collectively contribute to the pathogenicity and clinical significance of Streptococcus pneumoniae as a leading cause of respiratory tract infections and invasive diseases.

**Figure 11***: Streptococcus pneumoniae are visible in this digitally colorized photomicrograph of the fluorescent processed sample, illustrating the diplococcus appearance of two lance-shaped cocci.*

*Source: Public Health Image Library, Center for Disease Control and Prevention, Dr. M.S. Mitchell, 1964.*

**Phenotypic and biochemical properties:** *S. pneumoniae* is catalase-negative, meaning it lacks the enzyme catalase, which distinguishes it from catalase-positive bacteria like *Staphylococcus aureus*. It exhibits alpha-hemolysis on blood agar, characterized by a greenish discoloration around colonies due to partial lysis of red blood cells. Optochin sensitivity is a distinctive trait of *S. pneumoniae*, with the bacterium showing inhibition in the presence of optochin, aiding in its differentiation from other alpha-hemolytic streptococci such as *Streptococcus viridans* and *Streptococcus mitis*. Furthermore, *S. pneumoniae* is bile soluble, a unique feature that allows it to lyse in the presence of bile salts, facilitating its identification in laboratory settings. Lastly, *S. pneumoniae* is facultative anaerobic, capable of thriving in both aerobic and anaerobic conditions, reflecting its adaptability to various environments within the host.

**Culture conditions and media:** Luria-Bertani (LB) Agar in solid and liquid form is the most common media for culturing *S. pneumoniae*. Other well-known liquid media for this pathogen are Tryptic soy broth (TSB) and Todd Hewitt broth (THB). These selective media are frequently recommended for the cultivation of fastidious organisms such as *S. pneumoniae* to obtain the serotype specific capsular polysaccharides used as antigens for vaccine production (Suárez & Texeira, 2019; Kim et al., 1996). Defibrinated blood of sheep, horse, and even humans can be supplemented into the agar as well due to the high metabolic needs of the bacteria and supports hemolysis observation (Suárez & Texeira, 2019).

**Molecular identification:** *lytA* (major autolysin gene) and *piaB* (permease gene of the ABC transporter) were reported to be the target genes with markedly high specificity of 99.5% for the identification of *S. pneumoniae*. Another novel putative transcriptional regulator gene *sp2020* was recorded to possess even higher specificity (99.8%) (Tavares et al., 2019). Other genes with less specificity include *plyA* (pneumolysin), and *psaA* (lipoprotein component) (Sanz et al., 2018). Notably, different types of detection methods result in different specificity. One study found that identification of *lytA* and *blpA* using conventional PCR resulted in specificity of 87.71% and 89.47% respectively, while Real-time PCR gave significantly higher percentage with both 96.49% for *lytA* and *blpA* (Mosadegh et al., 2020).

**Virulence factors:** *S. pneumoniae* possesses several key virulence factors that contribute to its pathogenicity. Firstly, the polysaccharide capsule is antiphagocytic, inhibiting complement deposition on bacterial surface and can protect itself from being trapped in neutrophil extracellular traps. There are also surface proteins PspA, PspC, and LytA which act as adhesins to host cell surfaces, inhibit complement deposition (PspA), involve in biofilm formation and haemolytic effects of *S. pneumoniae* on human red blood cells (LytA). Furthermore, metal-ion-binding proteins PsaA, PiaA, and PiuA are involved in iron uptake and play a role in promoting opsonophagocytosis of *S. pneumoniae*. Other enzymes include autolysin, pneumolysin, IgA protease are crucial pneumococcal-protein virulence factors as well (Aryal, 2022; Kadioglu et al., 2008).

**Diseases and treatments:** *S. pneumoniae* is mostly known for the cause of community-acquired pneumonia, especially in children, the elderly, and immunocompromised patients. *S. pneumoniae* can also cause a wide range of illnesses, from moderate infections like otitis media (middle ear infection) and sinusitis (sinus infection) to potentially fatal infections of the central nervous system like meningitis. Aside from mucosal infections, it also has the ability to infect the bone, resulting in osteomyelitis, the joint system, causing septic arthritis, and the blood, causing bacteremia. Interestingly, *S. pneumoniae* is detected frequently in patients with Overwhelming post-splenectomy infection (OPSI) (42%) (Theilacker et al., 2015). Primary antibiotics treatments for *S. pneumoniae* are β-lactam antibiotics including penicillins and cephalosporins, macrolides, and fluoroquinolones. However, due to the increasing resistance to β-lactam and macrolides, fluoroquinolones which possess the lowest rate of resistance are highly recommended. Another option can be vancomycin, although it may become resistant in the near future (Zahari et al., 2023).

**Prevalence:** The prevalence of young children infected with *S. pneumoniae* is observably higher than adults. A study in Central Vietnam found that *S. pneumoniae* exists in 31.8% of children, and 23.9% of those had clinical pneumonia. Furthermore, the detection of *S. pneumoniae* was greater in the age groups of 0–1 and 2-3 years, and lowest in children older than five years old. (Wambugu et al., 2023). In a study with children under 5 years old in Ethiopia, the overall prevalence of *S. pneumoniae* in is 18% (CI 95%) (Mekuria et al., 2023), while another paper reported to identify 40.7% cases infected with this pathogen among community-acquired pneumonia patients in the UK (Lansbury et al., 2023). Some of the most identified serotypes in these cases are 6A/B, 19F, and 23F (Wambugu et al., 2023; Mekuria et al., 2023).

**STREPTOCOCCUS PYOGENES**

*Streptococcus pyogenes* belongs to the Lancefield serogroup A and is often referred to as Group A Streptococcus (GAS).

**Morphological characteristics:** *Streptococcus pyogenes*, a gram-positive bacterium, presents a distinct morphological profile characterized by its round shape and tendency to form chain-like structures. This pathogen lacks flagella resulting in its non-motility, and it does not form spores. Notably, this pathogen possesses a protective capsule primarily composed of hyaluronic acid, differentiating it from other encapsulated bacteria. This capsule provides protection against macrophage engulfment. Moreover, *S. pyogenes* has fimbriae, which are hair-like protein structures on its surface, aiding in adherence to host cells and biofilm formation.

**Figure 12***: A digitally regenerated image of Streptococcus pyogenes was created with scanning electron microscopic (SEM).*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC)/ Antibiotic Resistance Coordination and Strategy Unit, 2013.*

**Phenotypic and biochemical properties:** *S. pyogenes* lacks the catalase enzyme, a characteristic feature that sets it apart from catalase-positive organisms. Notably, *S. pyogenes* displays beta-hemolysis on blood agar, indicative of its ability to completely lyse red blood cells. And the reason for the lysis of red blood cells is because of an enzyme called streptolysin O (SLO) which interacts with the cholesterol membrane on red blood cells and completely lyses them (Duncan & Schlegel, 1975). Its sensitivity to bacitracin further aids in its identification, as it cannot survive in the presence of this antibiotic. Moreover, *S. pyogenes* is Pyrrolidonyl Aryl Sulfatase (PYR) positive, a trait that differentiates it from other beta-hemolytic enterococci, serving as a crucial diagnostic marker in clinical microbiology.

**Figure 13***: The right plate is Group A Streptococcus pyogenes (GAS), a typical beta-hemolytic bacteria with clear zone of red blood cell lysis, while the left plate is Streptococcus mitis, an alpha-hemolytic bacteria. Two petri dishes of trypticase soy agar medium both contain 5% defibrinated sheep blood.*

*Source: Public Health Image Library, Center for Disease Control and Prevention (CDC), Dr. Richard R. Facklam, 1977.*

<https://phil.cdc.gov/Details.aspx?pid=8172>

**Culture conditions and media:** Selective culture media commonly used for the growth of Streptococcus pyogenes include Columbia CNA agar with colistin and nalidixic acid, Phenylethyl Alcohol (PEA) agar, and Tryptic soy agar (TSA) with 5% defibrinated sheep blood (Spellerberg & Brandt, 2016). Columbia CNA agar has two antibiotics—nalidixic acid and colistin—that prevent gram-negative bacteria such as *Enterobacteriaceae* and *Pseudomonas* species from growing, and favor gram-positive bacteria like *Staphylococci, Streptococci*, and *Enterococci*. Likewise, PEA agar is selective for *S. pyogenes* due to the inhibitory effect of PEA on gram-negative bacteria and fungi, while allowing the growth of gram-positive organisms.

**Molecular identification:** *spy1258* is highly specific and is the most commonly used gene for the detection of *S. pyogenes*. Studies have shown that the *spy1258* PCR assay has the specificity of 93.85% to 100% for detecting the pathogen, as it only amplifies DNA from *S. pyogenes* and not from other *Streptococcus* species or common bacteria. Besides *spy1258*, genes encoded for virulence factors are also utilized as target genes such as *dnaseB, speB, and sof*. However, the specificity of these genes is relatively poorer than *spy1258* (Abraham & Sistla, 2016). Molecular identification method employed is usually PCR.

**Virulence factors:** *S. pyogenes* expresses a wide range of virulence factors from the unique capsule and surface-associated proteins to secreted enzymes and toxins. A hyaluronic capsule of *S. pyogenes* protects the pathogen from phagocytosis by the host immune system's neutrophils, while M protein embedded on the cell wall facilitates attachment to host cells and inhibits opsonization by the alternative complement pathway. Other components on the cell wall such as lipoteichoic acid and protein F (Sfbl), which is fibronectin-binding protein, further promotes epithelial host cell attachment. Moreover, some of the secreted virulence factors include Streptolysin O (SLO) and streptolysin S (SLS), which are hemolysins that lyse red blood cells, streptokinase which activates plasminogen to plasmin, aiding in tissue penetration, as well as hyaluronidase that breaks down hyaluronic acid in host tissues (Barnett et al., 2022). There are also Streptococcal pyrogenic exotoxins SpeA and SpeC which cause rash of scarlet fever and many other symptoms of Streptococcal Toxic Shock Syndrome (STSS) (Earhart et al., 2000).

**Diseases and treatments:** *S. pyogenes* can cause a wide range of diseases, from non-invasive illness such as pharyngitis (sore throat) and impetigo (mild skin infection), to severely fatal invasive infections such as necrotizing fasciitis, pneumonia, bacteremia, and Streptococcal Toxic Shock Syndrome (STSS). Necrotizing fasciitis is a deadly skin and soft tissue infection famously known as “flesh-eating disease", infected patients have a high fatality rate of 20-80% (Wallace & Perera, 2023). Streptococcal Toxic Shock Syndrome (STSS) is a rare disease defined by a series of acute symptoms including sudden onset of shock, organ failure, low blood pressure and in some worst cases, death. For pharyngitis, the recommended drug is penicillin. For fatal illnesses, it is advised to treat with vancomycin or clindamycin, along with removal of necrotic tissue in necrotizing fasciitis patients (Kanwal & Vaitla, 2023).

**Prevalence:** The prevalence of *S. pyogenes* among acute pharyngitis patients in Northwest Ethiopia is 9.1% which is comparatively lower than other regions such as Jimma, Ethiopia 11.3%, India 5.5%, Japan 5.8%, Indonesia 13.5% and Nepal 9.2%. However, it was higher than studies from Mexico (0.04–0.42%), Brazil (3.9%), Romania (4%), Iran (2.5%), and Saudi Arabia (1.5%). Nonetheless, studies have recognized the prevalence of this bacteria is 16-45% in African, 28.6-37% in USA, 30% in Iran and 69.5% in Israel (Kebede et al., 2021).This comes to show that the detection of *S. pyogenes* depends on sample sizes, geographical features, climate conditions at the sample collection period, as well as the detection method. Notably, pharyngeal infection in children older than three years old is higher than other age groups due to the exposure with the pathogen in schools, playgrounds, etc., and naturally weaker immune system in children (Efstratiou & Lamagni, 2022).

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