COMPARATIVE ASSESSMENT OF DIFFERENT LABORATORY TECHNIQUES (CONVENTIONAL, API AND VITEK) USED FOR THE CHARACTERIZATION OF GRAM-POSITIVE COCCI AT LAGOS STATE UNIVERSITY TEACHING HOSPITAL (LUTH).

CHAPTER ONE

1.0 INTRODUCTION

Taxonomy is an indispensable topic within the discussion of microorganism identification, as it is general knowledge that identification is a part of taxonomy. As described by Guerra-García et al. (2008), the word taxonomy is derived from the Greek words *taxis* (arrangement) and *nomos* (law). It is the subject of science that deals with the description, classification, and inventory of life (Wägele, 2005; Guerra-García et al., 2008). Aristotle's proposal of the first classification of living organisms began the subject of taxonomy. Thereafter, Linnaeus introduced the binomial classification/nomenclature which is in use till date and this classification has been most responsible for most of the recent taxonomic classifications (Engho, 2009). Characterization of Gram-positive bacteria is one of the most important but also routine tasks performed in clinical microbiology. In clinical laboratories the present means of identification of gram-positive cocci mainly rely on phenotypic tests. These tests have been miniaturized and semi-automated, leading to major progress in diagnostic accuracy (Kikuchi et al., 1995). Conventionally, the morphological and biochemical method of identification of bacteria is used in bacteria characterization. Classical identification of individual bacterial species in environmental/biological samples typically involves isolation, laboratory culture and then taxonomic characterization. (Lippincott Microbiology: Review of Medical Microbiology by Warren Livenson)

In classifying Gram-positive bacteria, their cell shapes are taken into consideration and this classification is divided into cocci, bacilli or branching filaments. Furthermore, in order to carry

out successful identification criteria including colony morphology, cell morphology, Gram reaction, oxygen requirements for growth, carbon source utilization, presence of endospores in a culture, motility The Gram-positive cocci of medical importance included in this review include *Staphylococcus* and *Streptococcus.* According to Simona et al., when common microorganisms present with uncommon phenotypes, when unusual microorganisms are not present in reference databases, or when databases are out of date, reliance on phenotypes can compromise accurate characterization (Simona et al., 2015).

The classification of bacteria into families, genera and species is based on a wide range of phenotypic characteristics (Juiz et al., 2012) including culture conditions, colony morphology and biochemical characteristics. In Nigeria, most of the Microbiological laboratories depend on the conventional methods to identify and study the diversity of bacteria. While these methods have a high usage rate among clinical laboratories, the characteristic features vital to these conventional methods are not static, they can change with stress or evolution (Mussap et al., 2007).

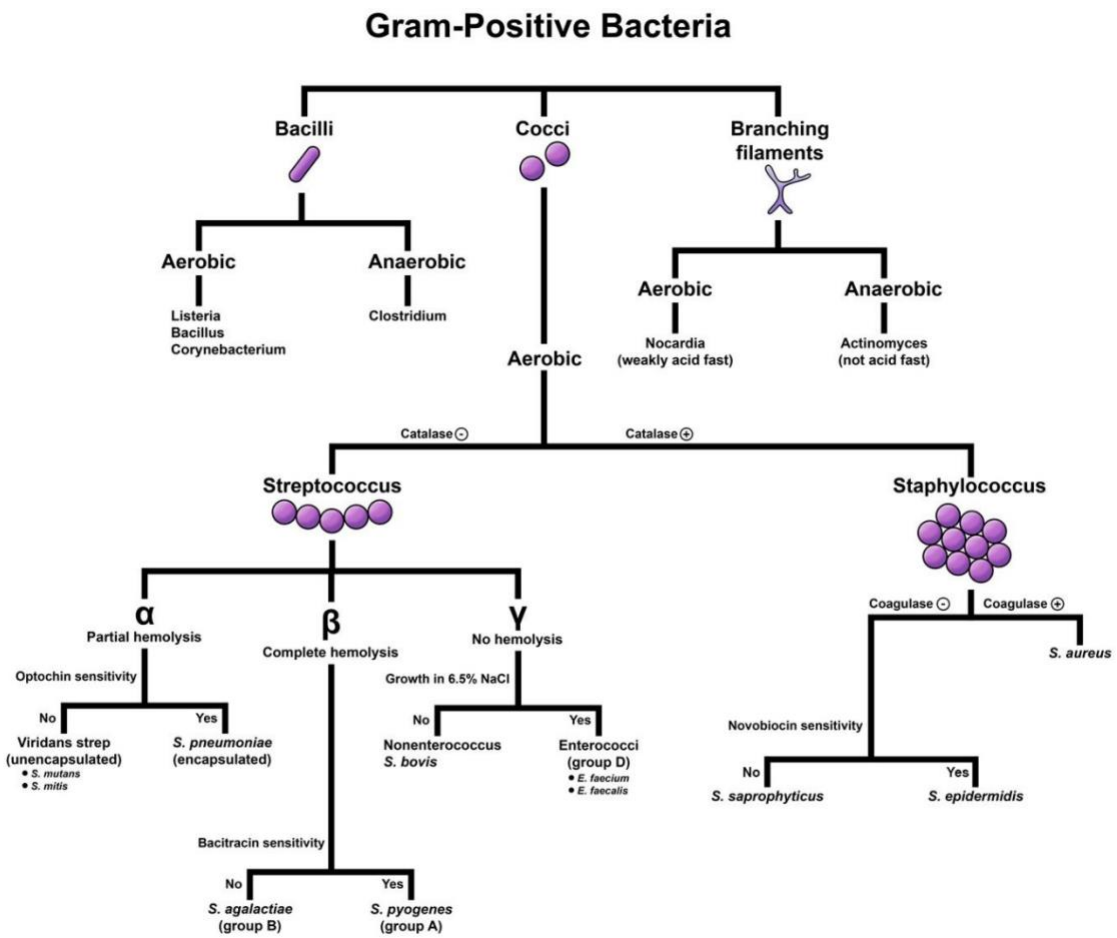


Figure 1. Classification of Gram-Positive Bacteria

The current pillar of laboratory diagnosis for most clinical infections is culture-based (solid media or broth) isolation the bacterium from patient samples using a plate-based method or a continuous monitoring blood culture system for bloodstream infections. (Buchan et al., 2013) Upon culture positivity on solid media, typically after 18 – 24 hours of incubation, a primary Gram stain is performed prior to biochemical testing of isolates to arrive at a conclusive bacterial identification. Following this, an additional 12–24 hours of incubation is required for antibiotic susceptibility testing. The time needed for microorganism identification using these traditional methods is approximated to run for 2-5 days at the least. (Franco-Duarte et al., 2019) This delay between confirmation, final identification, and antibiotic susceptibility results affords the clinician with insufficient actionable information during a critical phase of an infection. This leads to routine

treatment of patients with empiric broad spectrum antimicrobials which may not always be effective (Dellinger et al., 2008). This shortcoming of the conventional identification and diagnostics methods thus warranted the development and employment a variety of technologies in order to reduce the waiting gap between the different stages involved in identification and diagnosis. Precise identification of microorganisms is a critical task clinicians and scientists generally. There are several criteria considered in categorizing the plethora of methods used in the identification of microorganism, however, they can generally be classed as direct and indirect techniques (Table 1).

Table 1. Methods used in the area of microorganism identification.

Type	Basis
Direct	**Culture-independent Methods.** May be used to identify specific microbes in a mixed population as well as identify non-culturable microbes. For example, microscopic techniques are powerful tools used in the identification of microorganisms by visualization of the characteristic structures and for organisms in the VBNC (viable but not culturable) state.
Indirect	**Conventional methods.** Isolation and culture of microorganisms and the determination of their various phenotypic characteristics

Several advanced methods and systems including molecular methods, VITEK 2, fluorescent in situ hybridization (FISH), and more recently matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) among others have all been employed in the rapid identification of different Gram-positive cocci bacteria directly from clinical samples to species level. (Ligozzi et al., 2002; Wolk et al., 2009; Deck et al., 2012; Buchan et al., 2012). The turn-around-time of identification has significantly reduced with the introduction of these methods compared to routine culture. Additionally, the sensitivity and specificity of these advanced methods are relatively higher, with each method recording ≥90% (Buchan et al., 2013). However, some of these current methods- molecular methods and FISH are only able to detect and identify one or few specific targets while MALDI-ToF MS has the potential to detect nearly any bacterial species present in a clinical sample. Yet, when standard protocols were used, satisfactory confidence score results were recorded for only 67% - 80% of cultures containing Gram-positive bacteria (Stevenson et al., 2010; Buchan et al., 2012). Furthermore, the use of MALDI-ToF MS in differentiating *S. pneumoniae* from *S. mitis* is still an area of ongoing investigations. Progressively, scientists have provided solutions to these inadequacies largely through optimization of spectral analysis software and modification of scoring thresholds (Werno et al., 2012).

Each of these methods has its strengths and weaknesses. Consequently, the most recent research approach involves the use of a compilation of multivariate techniques, and this introduction appears to have great potential for the future. In order to obtain the most precise identification, classification, and systematics of microorganisms, it is extremely important to choose appropriate techniques, as well as have an exhaustive understanding of the mechanisms of their action. Therefore, this literature review is aimed at providing a description of the traditional and novel methods of identification (Table 2), as well as their strengths and limitations.

Table 2. Different techniques used for identification of microorganisms

Chromogenic Media	**Microscopy Techniques**	**Biochemical Techniques**	**Molecular Techniques**
Traditional Media	Bright field Darkfield SEM TEM CLSM ATM Inverted Microscopy	Traditional: • Media • API • VITEK • BBL Crystal Mass Spectrometry • GC • LC • MALDI-TOF • ESI	PCR RT-PCR Ribotyping Whole Genome Sequencing MALDI-TOF MS PFGE

1.1 Gram-Positive Cocci

Gram-positive cocci are one of the principal human bacterial pathogens known, comprising primary pathogens such as *Staphylococcus aureus, Streptococcus pyogenes*, and *Streptococcus. pneumoniae*, and species of lesser virulence including *Staphylococcus epidermidis*, *Staphylococcus Saprophyticus*, and *Enterococcus faecalis*. The detection and identification of this group of organisms is a vital but also routine tasks performed in clinical microbiology laboratories. An important clinical as well as taxonomic division in this group is the broad division into the Streptococcaceae and the Micrococcaceae based on colonial morphology, Gram morphology, and the catalase test. This chapter discusses the biology of two gram-positive cocci genus- *Staphylococcus* and *Streptococcus*. The *Staphylococcus* genus is a member of the family Micrococcaceae. They are Gram positive, with characteristic irregular grape-like clusters, tetrads, or short chains. They are catalase-positive, facultative anaerobes, nonmotile, non-spore-forming, and usually unencapsulated. On the other hand, the Streptococcaceae include Gram-positive spherical bacteria growing in chains or pairs. They are typically nonmotile, non-spore forming facultative anaerobes, and oxidase negative. They attack carbohydrates fermentatively and unlike the Micrococcaceae they are catalase negative, which is a distinguishing factor between the two families.

1.1.1 Genus *Staphylococcus*

Bacteria in this genus are known pathogens of man and other mammals. Traditionally they were divided into coagulase-positive staphylococci (CoPS) and coagulase-negative staphylococci (CoNS) on the basis of their ability to clot blood plasma (which is the coagulase reaction). The coagulase-positive staphylococci constitute *S. aureus* which is considered the most pathogenic species. The CoNS on the other hand are known to comprise over 30 other species. Although while

some CoNS species are able to cause infections, they are generally common commensals of skin. Coagulase adds more to the features of Staphylococci than being a major agent in the diagnosis and differentiation of Staphylococcus into CoPS and CoNS, it is also a virulence factor that causes cleavage of soluble fibrinogen to produce a fibrin coat in the surface of the bacteria. (Powers and Wardenburg, 2014). This serves as a protection mechanism for the bacteria against phagocytosis. Significantly, the coagulase coding gene, *coa*, is polymorphic, allowing it to be explored in molecular typing techniques (Javid et al., 2018). Nonetheless, as noted by Almeida et al. (2018), the applications of the coagulase method are quite limited because there are populations of CoPS that may lack the *coa* gene, while some CoNS express the gene, (Almeida et al., 2018). Strains with varying coagulase characteristics are often found in some species than others, but their misdiagnosis may cause inappropriate infection control and treatment (Rossi et al., 2020) The significance of this is particularly observed when the detection of the pathogenic *S. aureus* relies on coagulase production and the strains end up being non-coagulase producers, ultimately leading to misidentification (Sundareshan et al., 2017). Furthermore, studies have reported that strains of the CoNS *S. chromogenes, S. xylosus, S. cohnii* and *S. agnetis* can clot plasma, thus causing misidentification of the pathogens causing mastitis in dairy animals (Santos et al., 2016; Almeida et al., 2018). This has resulted in the reliance on more accurate identification methods, including the sequencing of the 16S rRNA and the housekeeping genes *rpoB*, encoding the β subunit of RNA polymerase, as well as *tuf*, encoding the EF-tu elongation factor (Li et al., 2012*).*

At least 30 species of staphylococci have been recognized by biochemical analysis and DNA-DNA hybridization. Of these 30, 11 can be isolated from humans as commensals, with 2 (*S. aureus* and *S. epidermidis*) (nares, skin) having the greatest pathogenic potential in humans. Other species of human infection include *S. saprophyticus* which is a common cause of urinary tract infections (UTI). *S haemolyticus, S simulans, S cohnii, S warneri and S lugdunensis* are also known to cause

infections in humans. While the basis of coagulase reaction is still in widespread use among clinical microbiologists, there are evidences that using this division can be misleading in some cases. (Foster, 1996) Additionally, coagulase is a known marker for *S. aureus* nevertheless there is no direct evidence that it is a virulence factor. Also, some natural isolates of *S. aureus* are defective in coagulase. Beyond being a major cause of monomicrobial infections, bacteria in the genus *Staphylococci*, particularly *Staphylococcus aureus* and *Staphylococcus epidermidis* are known to interact cooperatively or antagonistically with other bacteria, viruses or fungi, haven been reported in cases of polymicrobial infections (Kumari & Singh, 2019).

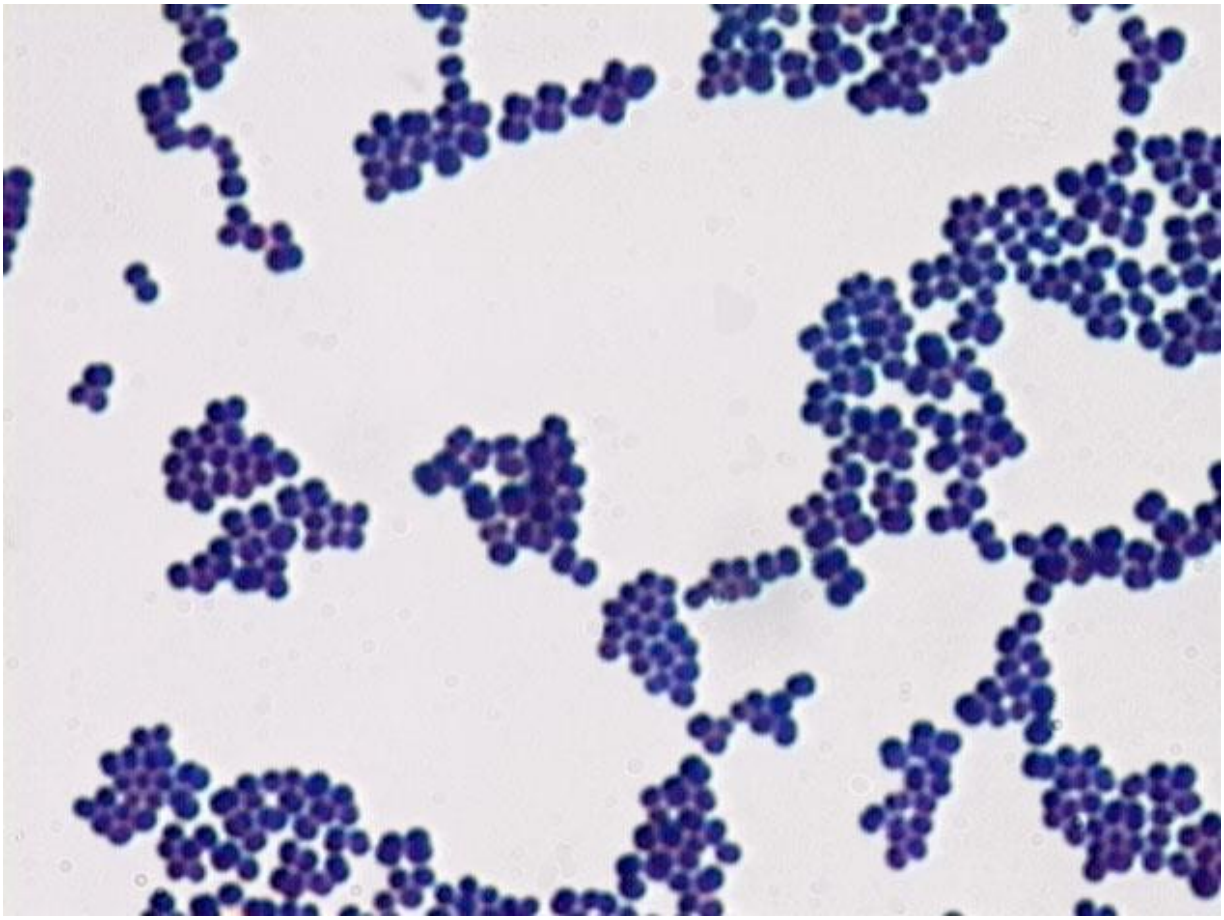


Figure 2. Microscopic view of a Gram-Positive Cocci

1.1.1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium and causative agent of several infectious diseases including skin infections, bacteremia, endocarditis, pneumonia and food poisoning. Originally, *S. aureus* was a leading cause of nosocomial infections. Subsequently, epidemiologically different clones emerged in community settings. (Gnanamani et al., 2016) *S. aureus* expresses an extraordinary repertoire of virulence factors that allows it to survive extreme conditions within the human host (Liu et al., 2009) The pathogen has incorporated in it the ability to acquire resistance to multiple antibiotics classes making it difficult pathogen to treat. In the last 22 years, there have been emergence of new clones of multidrug resistant *S. aureus* and wide spread methicillin-resistant *S. aureus* (MRSA), thus resulting in high morbidity, high mortality and increase in cost of treatment. (Liu et al., 2009) The emergence of these strains has limited the clinical utility of Vancomycin, which was the gold standard drug to treat the strains for many years.

Unlike traditional MRSA clones which are largely confined to healthcare settings and attack immunocompromised hosts, these new strains which are community-associated methicillin-resistant *S. aureus* (CA-MRSA) are known to infect previously healthy hosts, particularly children, young and middle-aged adults.

Microscopic morphology

S. aureus are characterized by Gram-positive cells that appear in spherical shape. The name 'Staphylococcus' was derived from Greek, meaning bunch of grapes (staphyle) and berry (kokkos) (Licitra, 2013) This is explained by the arrangement of the cells, which appear in clusters, when viewed under a light microscope after Gram staining (Gnanamani et al., 2016). Furthermore, roughly spherical shaped cells with smooth surface are revealed when observed under a scanning electron microscope while thick cell wall, distinguishing cytoplasmic membrane and amorphous

cytoplasm are shown when observed using the transmission electron microscopy (Touhami et al., 2004). Typically, the cells range from 0.5 to 1.0 μM in diameter.

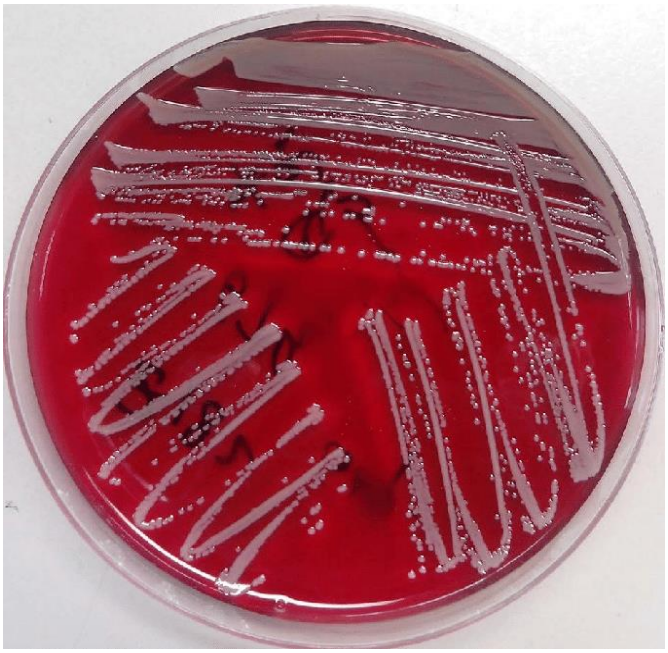


Figure 3A. Growth of *Staphylococcus aureus* after 24 hours on blood agar and aerobic incubation at 35°C

Figure 3B. Growth of *Staphylococcus aureus* after 24 hours on nutrient agar and aerobic incubation at 35°C

General cultural and biochemical characteristics

On nutrient rich agar medium, *S. aureus* produces either white colonies or yellow colonies. The yellow color of the colonies is typically imparted by carotenoids secreted by the organism. The bacterium is aerobic, facultatively anaerobic. (Gnanamani et al., 2016) and is often haemolytic on blood agar as a result of the production of haemolysins (alpha haemolysin, beta haemolysin, gamma haemolysin and delta haemolysin) (Dinges et al., 2000). The tolerance of the bacterium to salt enables it to grow on mannitol-salt agar medium that contains 7.5% sodium chloride (Brown et al., 2005). The organism is catalase positive and oxidase negative.

Virulence features

S. aureus presents several virulence factors that enable it to be successfully cause a plethora of both human and animal infections. The virulence factors of this pathogen help it to attach to host cells, break down the host immune defenses, invade tissues, cause sepsis and also elicit toxin-mediated syndromes. These pathogenic activities form the basis for a continued staphylococcal infection without strong host immune response (Kim et al., 2016). Table 3 shows the classification of *S. aureus* virulence factors based on their mechanism of action and role in its pathogenesis.

Traditionally, during laboratory diagnosis, the fundamental aim is to identify whether the diagnosed *S. aureus* isolate is resistant to methicillin (Gnanamani et al., 2016). A systematic diagnostic approach is necessary, since the emergence of MRSA, for early diagnosis so that appropriate antibiotic treatment can be started enough. Typically, slide and tube coagulase tests, latex agglutination tests and PCR-based tests are used for species level identification of this superbug. Additionally, determination of minimum inhibitory concentration (MIC) of antibiotic drugs including methicillin, oxacillin or cefoxitin using broth micro-dilution method, cefoxitin

disk screen, oxacillin agar screen and latex agglutination test for PBP2a and molecular methods for detection of *mecA* are used for the detection of MRSA (Brown et al., 2005).

Table 3. Virulence factors of S. aureus and its characteristics.

Virulence factors	**Activity**	**Characteristics**
Microbial Surface Components Recognizing adhesive matrix molecules (MSCRAMM)	Helping attachment to host tissues	They are cell surface proteins that interact with collagen, fibronectin & fibrinogen in the host, thus, enabling tissue attachment. Examples of these proteins include *Staphylococcal* protein A, fibronectin-binding proteins A and B, collagen-binding protein & clumping factor A & B. They also facilitate host immune evasion (Vazquez et al., 2011).
Polysaccharide microcapsule	Breaking/evading the host immunity	Resists killing by polymorphonuclear phagocyte (Nilsson et al., 1997).
Protein A		It binds to Fc portion of immunoglobulin and averts Opsonization. Also functions as a super antigen & restricts the host immune responses (Hong et al., 2016).
Alpha-toxin (Alpha hemolysin)		It is an exotoxin that causes pore formation in the cell membrane and ultimately leads to cell leakage & cell death (Gnanamani et al., 2016).
Chemotaxis-inhibitory protein of S. aureus (CHIPS)		An extracellular protein that impedes the chemotaxis functioning of monocytes as well as neutrophils (Postma et al., 2004).

Extracellular adherence protein (Eap)	Tissue invasion	An exoprotein which binds to host cell matrix, plasma proteins & endothelial cell adhesion molecule ICAM-1. In addition, it has immune-modulatory activity (Edwards et al., 2012).
Proteases, lipases, nucleases, hyaluronatelyase, phospholipase C, metalloproteases (elastase), & Staphylokinase.		These are extracellular enzymes that cause tissue damage and help in bacterial tissue penetration (Gnanamani et al., 2016).
Enterotoxins	Toxinosis Induction	Potent gastrointestinal exotoxins. The Staphylococcal food poisoning is an intoxication which results from consumption of foods containing sufficient amount of preformed enterotoxins (Argudin et al., 2010).
Toxic shock syndrome toxin -1 (TSST-1)		TSST-1 & some enterotoxins are regarded as pyrogenic toxin super antigens. It causes toxic shock syndrome especially in menstruating women (Dinges et al., 2000).

1.1.1.2 *Staphylococcus epidermidis*

Staphylococcus epidermidis is one of the most frequent causes of nosocomial infections, and it constitutively colonizes the human epithelial surfaces. It has been reported by epidemiological studies that healthy people carry between 10 and 24 different strains of *S. epidermidis* at a time (Kloos and Musselwhite, 1975) In addition to the abundance of species on the human skin, this high colonization rate is mainly due to the ability of the pathogen to adhere to the surfaces of medical devices during device insertion and form biofilms (Blaser and Falkow, 2009). Concurrent with advances in medicine, *S. epidermidis* has become the most common cause of primary bacteremia and infection of indwelling medical devices, particularly in immunocompromised individuals and neonates. (Fey and Olson, 2010) While there are also infections in sterile sites caused by this pathogen sterile site (e.g native valve endocarditis), most infections by the pathogen are associated with a foreign body such as catheter or other biomaterial (Rogers et al., 2009) Although biofilm production in this pathogen depends on multiple regulatory proteins (Li et al., 2005), it is still considered the most vital factor responsible for the establishment of *S. epidermidis* as a nosocomial pathogen (Li et al., 2009) Resistance to methicillin in *S. epidermidis* is another vital factor responsible for its establishment as a nosocomial pathogen. This resistance is known to be associated with the presence of the *mecA* gene, which encodes a penicillin-binding protein with low affinity for β-lactam antibiotics (PBP2A) (Goering et al., 2008)

Cellular morphology and biochemical characteristics

S. epidermidis is a resilient microbe, characterized by non-motility. As typical of Staphylococci, the cells are Gram-positive cocci that are arranged in grapelike clusters. The colonies, which are about 1–2 mm in diameter, are white, raised and consistent after 24 hours or overnight incubation.

and is not haemolytic on blood agar. It is a catalase-positive, coagulase negative, facultative anaerobe that can grow by aerobic respiration or by fermentation. Some strains may not ferment. Biochemical tests indicate this microorganism also carries out a weakly positive reaction to the nitrate reductase test. Although this bacterium can utilize glucose for growth anaerobically, production of coagulase and other agents like mannitol fermentation is negative, while in aerobic situation, the acid production occurs using different carbohydrates (fructose, maltose, sucrose, and glycerol).

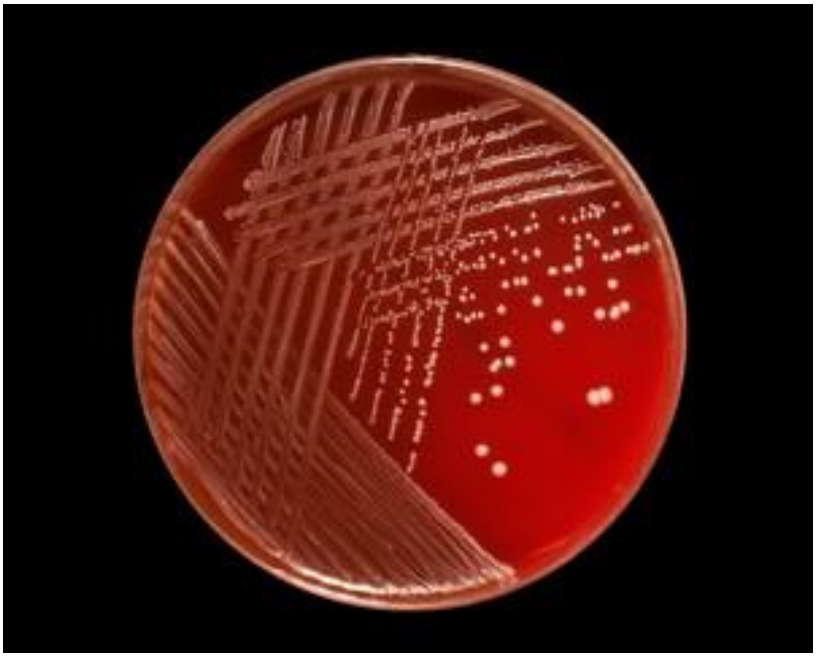


Figure 5A. Colonies of Staphylococcus epidermidis on sheep blood agar. Cultivation 24 hours, aerobic atmosphere, 37°C.

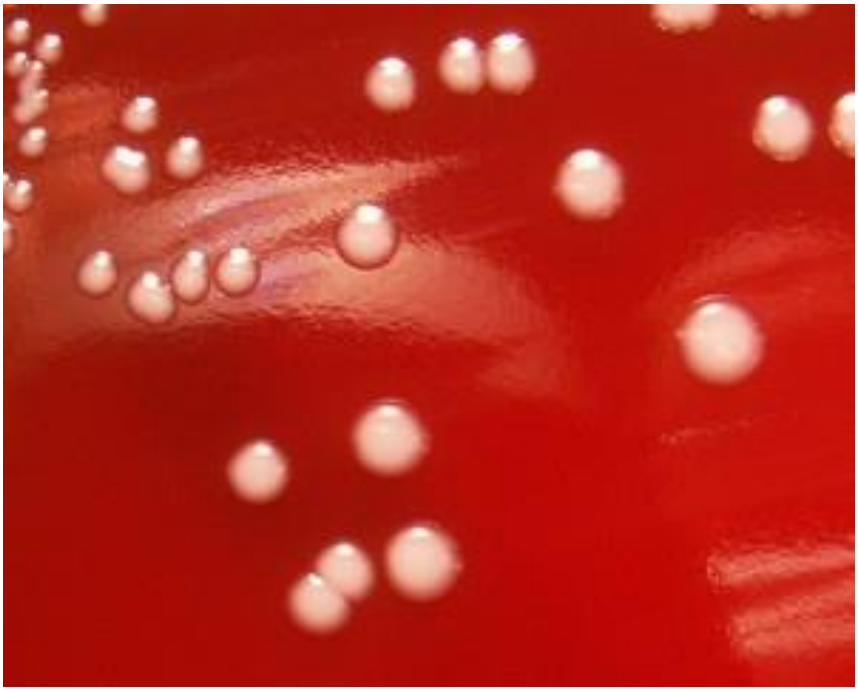


Figure 5B. Gamma-hemolytic (non-hemolytic) colonies of Staphylococcus epidermidis on sheep blood agar. Cultivation 24 hours in an aerobic atmosphere, 37°C.

Differentiating between *S. aureus* and *S. epidermidis* infections are important in the identification of highly contaminated and true bacteremia infections (Namvar et al., 2014). This ultimately calls for the rigorous and rapid diagnosis of the main cause of infection in clinical microbiology laboratories. Traditionally, studies in the past were commonly conducted on the basis of colony identification, culture medium, Gram staining, catalase test, coagulase and phosphatase activity, nitrate reductase, DNase, TNase, acid production from carbohydrates, tolerance to 10–15% NaCl, hemolytic activity on 5% blood sheep, antibiotic sensitivity test to polymyxin B and novobiocin for detection of CoNs specially *S. epidermidis* (Renneberg et al., 1995). In recent years, commercial kits for identification of *S. epidermidis* have been developed. These kits include API Staph-Ident, API Staph-Trac, Sceptor Gram-Positive MIC/I, Vitek GPI Card and Minitek Gram-Positive System. Rapid molecular methods such as peptide nucleic acid (PNA) and fluorescence in situ hybridization (FISH) now provide results in less than 2 hours. In QuickFISH method the coagulase-negative Staphylococci are determined in <30 minutes with blood culture containing tubes that specific probes are the advantage of this method. In many researches despite DNA hybridization and 16s rRNA analysis, ERIC and BOX-PCR have been used as a complementary method (Wieser and Busse, 2000).

Table 4 Significant virulence factors in *S. epidermidis*

Virulence factor	Description
Biofilms	Bacterial surface adhesive accumulation that is embedded in an extracellular matrix that creates the bacteria protection against host defense mechanisms and antimicrobial agents. Reduces cell permeability, cell division and protein synthesis. It also has anti-phagocytic activity and antimicrobial barrier function.
Biofilm associated protein	A surface adhesion protein commonly found in *S. epidermidis* strains.
Toxins	Enterotoxin-like toxin L (SEIL) and C3 enterotoxin (SEC3).
Phenol-soluble modulins (PSMs)	They are α helical peptides found in all pathogenic Staphylococci and are able to lyse the white and red blood cells. They also express cytokines, activate human neutrophils and inflammatory response.
Delta-Toxin	Responsible for hemorrhagic enterocolitis in the neonatal intensive care unit that can enhance the virulence potential of *S. epidermidis* and may lead to endemic and epidemic infections in different wards of hospitals.
Embp	An extracellular matrix binding protein which can mediate biofilm accumulation and fibronectin attachment.

Namvar et al., 2014

1.1.2 Genus *Streptococcus*

Streptococci are generally Gram-positive cocci. They are nonmotile, non-sporeforming, catalase-negative, and appear in pairs or chains. Most of the members in this genus are facultative anaerobes while some are obligate anaerobes. Most require enriched media (blood agar). Group A streptococci have a hyaluronic acid capsule. They naturally inhabit the oral cavity and intestines of both humans and animals. In addition, they often inhabit the skin, throat, and upper respiratory tract. However, numerous streptococci occur as opportunistic pathogens, causing infections in the case of weak immunological response of the host body (Krzyściak et al., 2013). This genus was one of the first groups of microorganisms to be identified as causing infectious diseases. It was the emergence of these pathogens that led to the introduction of hygiene and aseptic practices in hospitals (Nobbs et al., 2009). *Streptococci* classification was traditionally based on the Lancefield scheme- grouping of *Streptococcal* strains according to the carbohydrate composition of the antigens in their cell wall. These antigens are polysaccharides, teichoic acids, or lipoteichoic acid, and are generally known as group-specific antigens or C substances. Currently, their classification is based on colony morphology, hemolysis, biochemical reactions, and serologic specificity. They are divided into three groups by the type of hemolysis on blood agar: β-hemolytic (clear, complete lysis of red cells), α hemolytic (incomplete, green hemolysis), and γ hemolytic (no hemolysis). Serologic grouping is based on antigenic differences in cell wall carbohydrates (groups A to V), in cell wall pili-associated protein, and in the polysaccharide capsule in group B streptococci. Pathogenic species are grouped into: those commonly causing infections in humans, commensals and epizootic species, known to cause disease symptoms under specified conditions (Cole et al., 2008). *S. pneumoniae, S. pyogenes,* and *S. agalactiae* are typical pathogenic species in this genus.

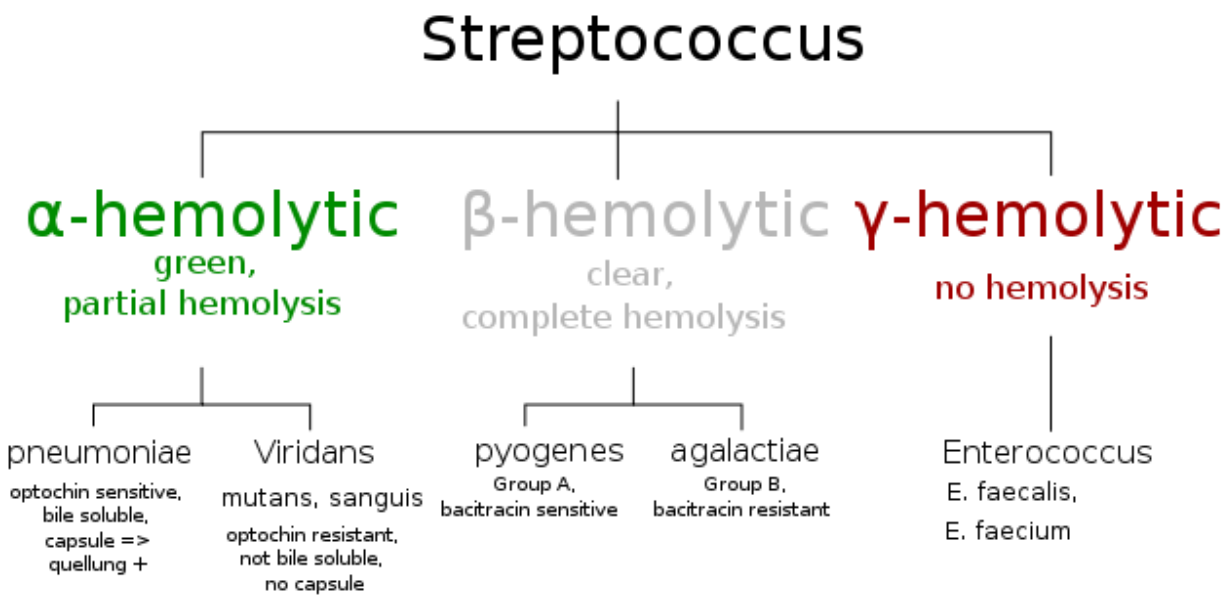


Figure 5. Streptococcus identification flowchart

1.1.2.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram-positive bacterium that is also known as *pneumococcus*. The bacterium survives in both aerobic and anaerobic conditions, and the cells often appear as diplococci (Bridy-Pappas et al., 2005). While different studies have reported a varying number of identified serotypes of *S. pneumoniae* (CDC, 2015; Geno et al., 2015; Sugimoto et al., 2017), there are over 97 serotypes that have been identified and characterized (Keller et al., 2016). The pathogen is currently one of the most important human pathogens, holding the record as a worldwide leading cause of bacterial pneumonia, meningitis, and sepsis. The bacterium is carried asymptomatically in the nasopharynx of healthy children, with an onset of colonization shortly after birth. It is also, less frequently, carried asymptomatically in the nasopharynx of healthy adults (García et al., 2010).

S. pneumoniae is a fastidious bacterium and it grows optimally at 35-37°C with ~5% CO_2. Growth media including blood agar and chocolate agar are typically used to culture the bacterium in the laboratory. On a blood agar plate (BAP), colonies of *S. pneumoniae* appear as small, grey, moist (sometimes mucoidal), and they typically produce a zone of alpha-hemolysis (green) (Figure 1). This alpha-hemolytic property is a differential factor between the bacterium and many other species. Once pneumococcal culture ages 24-48 hours, their colonies morphology changes, they become flattened, and the central portion becomes depressed. *S. pneumoniae* can be identified using Gram stain, catalase, and optochin tests simultaneously, with bile solubility is used as a confirmatory test. If these tests indicate that the isolate is *S. pneumoniae,* serological tests to identify the serotype can be performed.

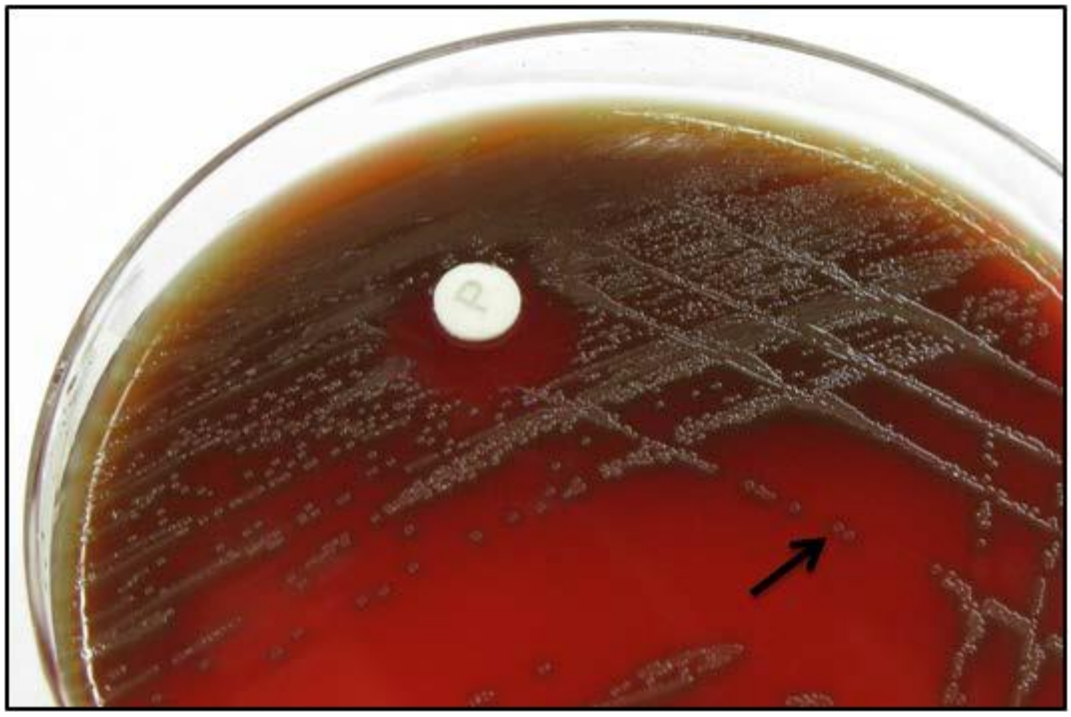


Figure 4 *S. pneumoniae* colonies with a surrounding green zone of alpha-hemolysis (black arrow) on blood agar plate

1.1.2.2 *Streptococcus pyogenes*

Also known as beta-hemolytic group A streptococcus or Lancefield's group A streptococcus (GAS), it is the most pathogenic specie in the *Streptococcus* genus. (Facklam, 2002). In the identification of *S. pyogenes* in clinical samples, colonies of the cultured isolates are checked for β-hemolytic activities on blood agar plates. Upon 24 hours of incubation at 35-37°C, the colonies of *S. pyogenes* appear dome-shaped with a smooth or moist surface and clear margins. A typical white-greyish color is observed in the colonies and they are usually > 0.5 mm in diameter. Microscopically, the cells of *S. pyogenes* appear as Gram-positive cocci that are arranged in chains. The detection of β-hemolytic colonies on blood agar plates, subsequent tests including catalase test are conducted to confirmation. Other rapid laboratory identification tests can then be applied for definite species identification (Spellerberg et al., 2016). Some of these tests are the Lancefield antigen determination which was developed to differentiate β-hemolytic *streptococci* into different species by determining the presence of Lancefield antigens on streptococcal surfaces through antibodies (Lancefield, 1933); the PYR test which is a rapid colorimetric method that differentiates *S. pyogenes* from other morphologically similar β-hemolytic *streptococci* and tests for the presence of the enzyme pyrrolidonyl aminopeptidase (Kaufhold et al., 1989); Bacitracin susceptibility tests which differentiate *S. pyogenes* from other non-group A β-hemolytic *streptococci* by their increased sensitivity to bacitracin; automated identification systems including MALDI-TOF (bioMérieux, Inc., 2015), Verigene Gram-positive blood culture (BC-GP) nucleic acid test (Nanosphere, Inc., 2014), and the Film Array platform for the direct identification of bacterial pathogens from blood culture bottles (BioFire Diagnostics, LLC, 2015; Altun et al., 2013; Wojewoda, et al., 2013)

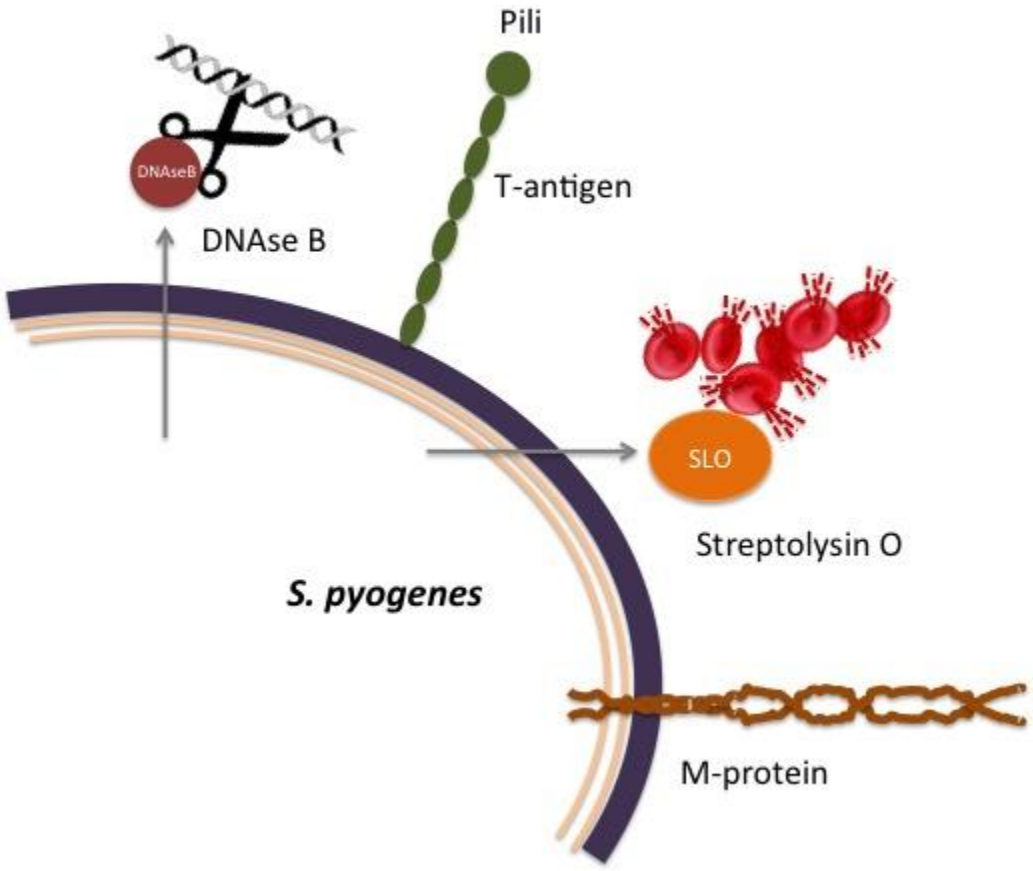


Figure 5. Common antigenic proteins of S. pyogenes used for diagnostic and typing purposes.

1.1.2.3 *Streptococcus agalactiae*

Streptococcus agalactiae which is also known as group B streptococcus or (GBS) is a Gram-positive coccus with a tendency to form chains (Whiley and Hardie, 2009). Generally, GBS is commensal that is known to be a normal flora of the human microbiota colonizing the GIT and genitourinary tract of up to 30% of healthy human adults (referred to as asymptomatic carriers). However, the pathogen can cause severe invasive infections especially in neonates, elderly people, and people with compromised immune systems (Edwards and Baker, 2010). It is beta-hemolytic, catalase-negative, and a facultative anaerobe. On blood agar plates, *S. agalactiae* readily grows with β-hemolytic colonies. In identifying this pathogen, latex agglutination test is one of the tests that is conducted, and it operates on the basis of detecting the antigen group B of Lancefield classification that is present in the cell wall of the bacterium (Tille, 2014; Rosa-Fraile and Spellerberg, 2017). Other important tests used in the identification of *S. agalactiae* in the laboratory include the CAMP test; presumptive identification based on the ability of S. agalactiae to hydrolyze Hippurate (Tille, 2014); Orange-brick-red pigmentation by hemolytic strains when cultivated on granada medium (Rosa-Fraile et al., 2006); MALDI-TOF instruments (To et al., 2019). In concert with the listed identification methods, appearance of *S. agalactiae* colonies on chromogenic agar media can additionally be used for tentative identification (Filkins et al., 2020).

Figure 8A. β-hemolytic colonies of *Streptococcus agalactiae*, blood agar after 18h of incubation at 36°C

Figure 6B. *Streptococcus agalactiae* on granada agar, anaerobic incubation.

1.1.2.4 *Enterococcus sp*

Enterococcus species are typical Gram-positive bacteria that make up part of the normal flora of the GIT of humans (Lance et al., 2009). Apart from inhabiting the human GIT, they are also found in fecally contaminated soil, sewage, water and food samples. *Enterococcus* are opportunistic pathogens and they cause serious diseases including of infective endocarditis, meningitis, pneumonia. Also, they are known to cause infections in surgical wounds, burn, skin and soft tissue, urinary tract, bone and joint (Kavindra et al., 2010). There are 16 species within this group and among them is *Enterococcus faecalis*, a pathogen that is reported to have the highest isolation rate (Li, 2006). The resistance profile and virulence factors of the pathogen confers on it the capacity to cause very serious infections. Over 10 types of virulence factors have been detected in clinical isolates and these virulence factors include haemolysin activator (*cylA*), gelatinase (*gelE*), *E. faecalis* surface proteins (*esp*), endocarditis antigen (*efaA*), collagen-binding protein (*ace*) and 2 surface proteins (ef0591 and ef3314) (Creti et al., 2004). Apart from being a serious problem in humans, *Enterococcus* infections pose an equal threat to animals, especially those in veterinary clinics (Stalker et al., 2003), and different clones have the ability to acquire several genetic elements that encode potential virulence factors, antibiotic resistance genes and genes that enable adaptation to harsh environments thought to enhance pathogen survival in hospitalized patients.

In terms of identification, a greater percentage of clinical microbiological laboratories primarily relies on phenotypic characterization. While this is true, several studies have revealed that an unambiguous species identification of *enterococci* using phenotypic methods is a painstaking procedure as it can take several days to achieve as a result of the phenotypic and biochemical similarities observed between many enterococci species (Cheng et al., 1997). Molecular genetic techniques, such as randomly amplified polymorphic DNA analysis, intergenic ribosomal PCR, or

other PCR-based methods targeting various genes, have been successfully used to identify enterococci at the species level (Kirschner et al., 2001).

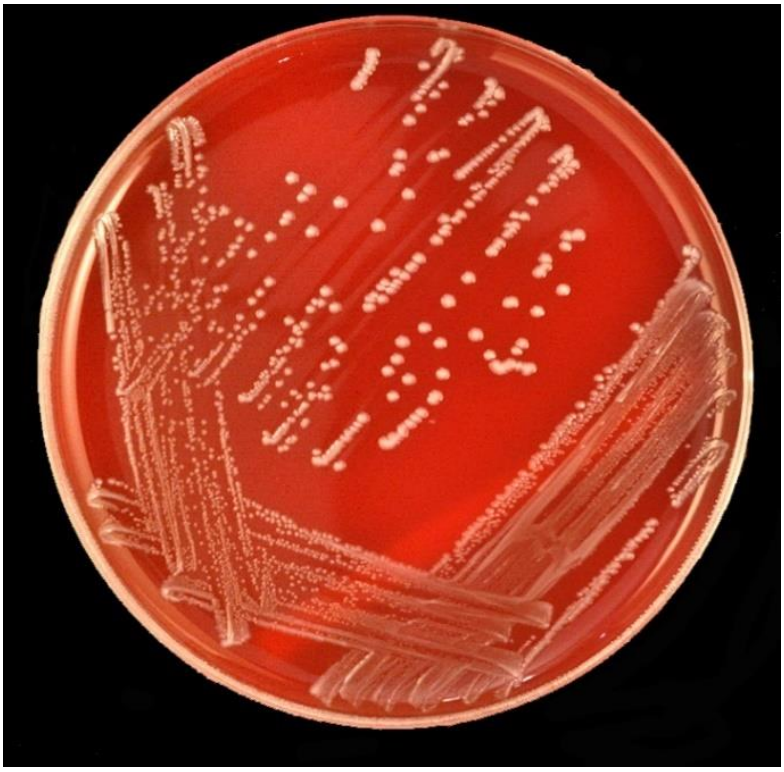


Figure 7. *Enterococcus faecalis* on blood agar plate

1.2 LABORATORY DIAGNOSIS OF GRAM-POSITIVE COCCI

1.2 Historical Evolution of Microorganism Identification

For many years, researchers pursued the opportunity of more swift and effective means of identifying microorganisms (Bisen et al., 2012) Prior to this and for many years, phenotypic method of classification was the only available means of identification. However, several uncertainties and difficulties characterized the method as described in different studies (Donelli et al., 2013). In the study of Yeung et al. (2002), typing discrepancies in the identification of *Lactobacillus acidophilus* and *Lactobacillus casei* using phenotype-based techniques were described. Traditionally, phenotypic methods of identification were based on dichotomic keys, with Gram staining being the first test to be performed. This was immediately followed by catalase and oxidase tests. As described by Lagier et al. (2015), Identification on the basis of carbohydrate metabolism was evaluated through acid production analysis, and end products of this metabolism including CO2, acetate, and lactate. The development of enzymes tests then followed this development sequence. (Lagier et al., 2015). Furthermore, tests for protein and amino acid metabolism including indole production, casein digestion, decarboxylation of lysine, ornithine, arginine, phenylalanine deaminase, and urease were developed, thereby supplementing other phenotypic tests such as tests for lipid metabolism, or the experiments based on cell wall receptors, including optochin, lysozyme susceptibility, and bile solubility tests, which are often used for the differentiation of Gram-positive cocci (Lagier et al., 2015). In recent years, several phenotypic features of different microorganisms can be simultaneously confirmed in less than 48 hours through the use of commercially available tools or automated phenotypic systems. (Franco-Duarte et al., 2019) As described by Skerman in 1959, phenotypic tests are Phenotypic tests are performed in a logical order, with each result indicating the next test to be done (Skerman, 1959). However, realizing that difficulties that could lead to false identification of microorganisms could arise,

Steel, in 1965, wrote “Difficulties in interpretation of keys arise where strains behave inconsistently in some respect Some characters are almost invariably positive or negative, but characters of such constancy are usually shared by similar organisms, and although they are important in characterizing an organism, have little value in distinguishing it from its neighbors.” (Steel, 1965). Consequently, molecular tools and sequence databases were developed. These methods, had significant impact and contributions to the expansion of the power of Microbiology and increased the number of known microbial species (from 1971 in 1980, to 12,391 in 2013) (Lagier et al., 2015). According to Madigan et al. (2012), molecular biology emerged in 1941, when DNA was discovered as the genetic material. The use of genetics as a tool for bacterial identification started in 1985 with the design of PCR (polymerase chain reaction). (Madigan et al., 2012) Several methods including culture dependent and culture independent approaches; and methods based on different principles such as DNA sequencing, 16S rRNA sequencing, and hybridization). Additionally, more advanced approaches and tools including the Omics tools-metagenomics, proteomics, lipidomics, transcriptomics, and metabolomics (Breitwieser et al., 2017), have emerged, which aim at a collective high-throughput characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of an organism or organisms (Klenk, 2019). This omics era ushered in the most recent advances of microbial identification, and it highlights a phenomenon similar to a rediscovery of past approaches and methods.

1.3 CONVENTIONAL METHODS OF IDENTIFICATION

In many clinical laboratories in Nigeria, the primary method of identifying gram-positive cocci are the phenotypic tests. However, phenotypic tests are characterized by potential inherent problems; e.g., (i) not all strains within a given species may exhibit a common characteristic, (ii) the same

strain may give different results upon repeated testing, and (iii) the test result relies on individual interpretation and expertise. Moreover, small alterations in the execution of an assay may give false test results. Consequently, identification based on phenotypic tests does not always allow an unequivocal identification.

1.3.1 Identification Methods Using Bacterial Culture

In these methods, the identification of microorganisms based on cultivation has the initial objective of obtaining pure culture. A pure culture containing a single type of microorganism can be obtained in various ways from enrichment cultures. Frequently used isolation methods include seeding by dewatering, deep seeding in solid media, and liquid dilution. For organisms that form colonies in solid medium plates, the technique of seeding by exhaustion is fast, easy, and the method of choice. From the repeated collection and seeding of an isolated colony, a pure culture can be obtained. By using appropriate incubation devices it is possible to purify both aerobic and anaerobic organisms in solid medium plates by using the seeding method by depletion. It is also important to point out that through cultivation, the microorganism can be identified from the production of certain metabolites released in the medium with lactic acid and others, and therefore the technique is based on the reaction of the medium with the released metabolites (Yang et al., 2019).

The *Staphylococci* and *Streptococci* are generally grown on agar media supplemented with blood such as blood agar (BA). This technique allows the detection of the hemolytic activities of the different species, which is important for subsequent identification steps. Additionally, selective media for cultivating Gram-positive bacteria (such as agar media that contains phenylethyl alcohol, or Columbia agar with colistin and nalidixic acid) are known to provide sufficient culturing conditions for species including *S. pyogenes* (Spellerberg and Brandt, 2016). The use of chromogenic agar media is also employed in isolating and identifying uropathogens including *Enterococci* spp. *Enterococci* is known to produce the enzyme ß glucosides, which react with ß

glucoside chromogenic substrate and will grow as distinct blue colonies on the agar media, hence providing apparent differentiation of the bacterium (Sharmin et al., 2010). However, culture-based identification alone is incomplete, since a distortion of the enrichment is a serious problem found with studies on biodiversity dependent on cultivation (Madigan et al., 2012). Furthermore, an issue as reported by Spellerberg and Brandt (2016) is the role of nonhemolytic S. pyogenes isolates particularly when culturing pharyngeal specimens for *S. pyogenes* on BAP. Culture based screening relies on the detection of β-hemolytic colonies and subsequent identification steps. However, clinical nonhemolytic strains of *S. pyogenes* have repeatedly been implicated as causing pharyngitis, as well as invasive infections (Jantsch, et al., 2013). Hence, standard culture methods will not detect these strains.

1.3.2 Microscopy Techniques

The microscope is an essential identification tool for microorganisms present in a sample (clinical or environmental). Images generated from the microscope facilitate the analysis of shape, tracking of motion, and classification of biological objects. The microscope-based observation is still a widely used method that is applied to definition of the morphological differences of bacteria species including *Streptococci* and *Staphylococci* both in clinical and research settings (Rajwa et al., 2010). While this method has its significance, there are challenges, including the difficulty of automated segmentation for many commonly used non-fluorescence, interference-based microscopy imaging modalities (Obara et al., 2013).

However, for many reasons, the use of microscopy alone to identify microorganisms is not sufficient. First, small cells usually present difficulty in identification, and they can be missed during the observation. Also, distinguishing between living and dead cells pose a great difficulty to microscopy (Madigan et al., 2012). Additionally, the inability of the microscopy method to reveal

phylogenetic relationship is a major limitation to it (Madigan et al., 2012). However, coordination of microscopic analysis with other tools fives the microscopy method more potentials. transmission electron microscopy (TEM); scanning electron microscopy (SEM), confocal microscopy (CLSM), and atomic force microscopy (ATM) are techniques of electron microscopy that are powerful instruments use for the identification of microorganisms (Cardinale et al., 2017). These techniques prove to be valuable mainly in the identification of microorganisms in biofilms including *Staphylococcus* and *Enterococcus* (Beier et al., 2012).

Generally, microscopic techniques are used together with fluorescent dyes, and this ensures specificity in visualization makes it easier to perform. DAPI (49,6-diamido-2-phenylindole) and acridine orange dye are examples of fluorescent dyes used widely. SYBR® Green I is another dye that gives bright fluorescence to all microbial cells, including viruses. These dyes bind to the DNA and fluoresce powerfully when exposed to ultraviolet (UV) rays at different wavelengths: DAPI at 400 nm, acridine orange at 500 nm, and SYBR Green I at 497 nm. This fluorescence causes the microbial cells to be visible (Sabnis, 2015). The specificity of these dyes to nuclei acids nullifies the occasional challenge of background coloring. Thus, making them usable for samples from many sources including soil and water. Although described a century ago, the Gram staining procedure remains the most frequently used rapid diagnostic test used in clinical laboratories to identify bacteria (Daniel, 2000). It is a key preliminary step in the initial identification of bacteria based on staining characteristics, facilitating visualization and examination of the bacteria using a light microscope. In an unstained smear, the bacterial cells are invisible when visualized under a light microscope. After staining the smear, the morphology and arrangement of the bacterial cells may then be observed. Furthermore, it is also an important step in the screening of infectious agents in clinical specimens such as direct smears from a patient. Although the Gram staining is used for detection and differentiation of bacteria, other microorganisms, most frequently yeasts and fungi,

can be seen on a Gram-stained smear (Barenfanger and Drake, 2001). Typically, the cells of Gram-positive cocci including *Staphylococci* and *enterococci* are stained dark purple because the cell wall of these bacteria retains the primary dye called crystal violet (CV).

1.3.3 Traditional Biochemical Methods

Traditional methods of identification in Microbiology rely to a great extent on the use of culture media. These methods still continue to be in use after many years, especially in laboratory routines involving a rapid identification of a microorganism. The typical phenotypic identification method usually functions on the basis of reactions of the microorganism to different chemicals (Franco-Duarte et al., 2019). One of the traditional methods most used is a simple visual detection of growth of the tested organism in the presence of a substrate by increased turbidity. Results are determined by comparing the microbial under analysis with a control test, and a Wickerham card is used to read the turbidity (O'Hara, 2005).

The analytical profile index (API) (bioMérieux, Craponne, France) is a typical model of identification used where standard methods are incorporated into miniaturized reaction couples, scored as "positive" and "negative" and finally matched to a scoring system on the basis of "best fit" (Sutton, 2007). API 20E (analytical profile index 20E) testing apparatus is a system that comprised a plastic piece with 20 cupules containing pH-based substrates that facilitate bacteria identification (Sandle, 2014). In an attempt to reduce the time, there were improvements to the API 20E system (O'Hara, 2005). The Vitek® system (bioMérieux, Craponne, France) is useful for simultaneous bacterial identification and antimicrobial susceptibility testing (AST) profiles (Puttaswamy et al., 2018). The system uses a totally automated broth microdilution technique that applies attenuation of light measured by an optical scanner for growth or no growth detection (Ligozzi et al., 2002).

1.4 IDENTIFICATION WITH THE API (ANALYTICAL PROFILE INDEX) 20 SYSTEM

API identification products are test kits used for identifying both Gram-positive and Gram-negative bacteria, as well as yeast. API strips give accurate identifications based on extensive databases and are standardized, easy-to-use test systems. The kits include strips that contain up to 20 miniature biochemical tests which are all quick, safe and easy to perform. API Staph system identifies clinically important staphylococci and micrococci overnight; RAPIDEC® Staph identifies commonly occurring staphylococci in 2 hours; and the API 20 Strep identifies streptococci and enterococci within 4 – 24 hours.

Principle

The API 20 Strep strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to reconstitute the enzymatic substrates. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The fermentation tests are inoculated with an enriched medium which rehydrates the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator. The reactions are read according to the Reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

CHAPTER TWO
LITERATURE REVIEW

2.1 TRADITIONAL CONVENTIONAL METHOD

For many years, established phenotypic tests have remained cornerstones for the identification of bacterial microorganisms in clinical microbiology. Various test systems thatintegratemultipleconventionalphenotypictestsintoasingle-stepprocedurehave been developed. After the interpretation of results, the biochemical reactions included in the kit system generate a biotype number that is matched against the profile indexes from a database to identify the bacterial species. Examples of biochemical profiling kits for the identification of streptococci are the API Rapid Strep identification system (bioMérieux) and the RapID STR system (Remel). However, the ability of these systems to identify microorganisms depends on the accuracy of their databases of profile indexes and the inclusion of all relevant microbial species. Although these systems can be used to identify beta-hemolytic streptococci, they are not 100% specific. As Winn and colleagues noted, while the accuracy to identify GBS is high, more reliable results may be achieved by using a combination of simpler phenotypic methods, reserving the use of these kits for the identification of other streptococcal species (Winn et al., 2006).

Most human Group B Streptococcus (GBS) isolates easily grow on blood agar after overnight incubation, as large colonies (3 to 4 mm in diameter) with a narrow zone of beta-hemolysis (Whiley and Hardie, 2009). While GBS beta-hemolysis may be difficult to detect for some strains, it can be observed only when colonies are detached from the blood agar. In most laboratories, colonies displaying typical GBS morphology are subjected to latex agglutination tests to determine the presence of the Lancefield group B antigen and, if they test positive, they are designated GBS. A decision that is based on detection of the Lancefield group B antigen is very specific, since *S.*

agalactiae is the only streptococcal species harboring this antigen. However, *Streptococcus porcinus*, which may be present in the genital tract of pregnant women, grows on blood agar as beta-hemolytic colonies and cross-reacts with GBS agglutination kits. For this reason, the detection of beta-hemolytic colonies that are positive in GBS latex agglutination tests requires further testing (Suwantarat et al., 2015). The zone of beta-hemolysis surrounding the *S. porcinus* colonies on sheep blood agar is usually wider and much more pronounced than that observed with *S. agalactiae* (Duarte et al., 2005). A simple and specific method to distinguish GBS from other beta-hemolytic streptococcal species is detection of the reddish polyenic pigment granadaene. The expression of granadaene is invariably linked to the expression of GBS β-hemolysin because they are encoded by a single genetic locus known as the cyl operon (Spellerberg et al., 2000). Moreover, it has been reported that GBS pigment and β -hemolysin could be the same molecule (Whidbey et al., 2013). As no other streptococcal species produce granadaene (Rosa-Fraile et al., 2014), detection of the pigment can be used as a simple and fully specific method for single-step identification of GBS, eliminating the need for ancillary tests. Detection of GBS pigment is generally carried out on Granada-type media, on which beta-hemolytic strains of GBS produce pigmented colonies. A folate pathway inhibitor (methotrexate) and Bacto Proteose Peptone N3 (BD) in the presence of starch trigger the production of pigment in these media (Rosa-Fraile et al., 2014).There are two classes of Granada-type culture media. The first is Granada agar, marketed in the United States by Hardy Diagnostics and in the European Union by bioMérieux and BD. The second is Granada broth, marketed by bioMérieux (ChromID Granada Biphasic) and in the United States by Hardy Diagnostics (Strep B Carrot broth). For optimal production of pigment, it is necessary to incubate Granada agar plates anaerobically or with a coverslip placed over the inoculum. However, anaerobic incubation is not necessary when using Granada broth. Although Granada-type media incorporate selective agents (crystal violet, colistin, and metronidazole), they

are not completely selective for GBS. Other microorganisms, mainly enterococci and yeast, can also grow on the medium and appear as white colonies. The identification of GBS strains by detection of hemolysis and pigment production is not 100% sensitive, however, because 2 to 5% of human-colonizing GBS isolates are non-hemolytic and do not produce pigment (Brimil et al., 2006). For such GBS strains, which appear white on Granada-type media, identification has to rely on other tests. Because β-hemolysin represents an important GBS virulence factor, strains lacking pigment and hemolysin production may not have full pathogenic potential (Whidbey, 2015). Since nonhemolytic and nonpigmented GBS strains are more frequently encountered among animal strains, Granada-type media are unreliable for the detection of GBS in veterinary clinical samples (Rosa-Fraile et al., 2014).

In a study by Capurro et al. (1990), in which a total of 414 coagulase-positive staphylococcal strains were identified using different traditional methods, ninety-seven per cent (97%) of the total number of strains identified were identified as *Staphylococcus aureus*, 2% as *Staphylococcus intermedius* and 1% as *Staphylococcus hyicus*. Two hundred and thirty seven (237) strains with atypical hemolysis reactions on bovine blood agar were randomly selected, with the aim to increase the number of *S.intermedius* and *S.hyicus* strains available for testing. Eight different characteristics, including physiological, enzymatical and biochemical properties, were used to identify the coagulase-positive Staphylococcus species. The findings in the study suggested that hemolytic reaction on chocolate agar is a reliable method in the identification of coagulase-positive Staphylococcus species.

2.1 ASSESSMENT OF API

The API20E system (API; bioMérieux, France) is a plastic strip with microtubes containing dehydrated substrates, originally designed for the identification of Enterobacteriaceae so that identification of fermenters with the system would be straightforward. The API20E system was extended to include non-fermenters by the addition of six supplementary tests and prolonged incubation of the API20E tests. Different researchers have assessed the API20E system for the identification of Enterobacteriaceae, reporting a high level of agreement with conventional methods in both biochemical reactions and identifications (Brooks et al., 1974; Nords et al., 1974). The performance of the API20E system in the laboratory depends largely on following the manufacturer's instructions namely; use of colonies from a pure culture, making a homogeneous bacterial suspension, proper inoculation into the cupules and incubating in the recommended environment and duration. Additional tests are done when the seven digit profile is not discriminatory enough. Review of records from the year 2006 to 2010, with a total of 1658 entries, showed One thousand four hundred and fifty two (87.6%) isolates to have exact identity, 199 (12%) nearest identity, and 7 (0.4%) no identity (Maina et al., 2014). From the review, it is obvious that API20E has performed reasonably well giving an exact profile in 87.5% of the isolates tested, a nearest profile in 12%, and <0.5% having no profile. As noted by Holmes and Dawson, there could be many causes of not obtaining an exact profile such as failure to follow the manufacturer's instructions on the period of incubation or the performance of supplementary tests, or both (Holmes and Dawson, 1985).

Different studies have compared the accuracy of the API20E system with other identification platforms. In a study by O'hara et al., after the initial incubation, 194 (77.0%), and 213 (84.5%) strains were correct to the genus and species levels with the API, and Vitek respectively. After additional biochemical tests were performed, as directed by each manufacturer's protocol, the

numbers of strains correctly identified to the genus and species levels were 241 (95.6%), 234 (92.8%), and 243 (96.4%)with the three systems, respectively (O'hara et al., 1993). Robinson and colleagues showed that of 381 isolates from the family Enterobacteriaceae, API20E and Crystal correctly identified 90.3 and 91.6% by 18 to 24 hours without supplemental testing, respectively, and Vitek identified 92.4 and 96.1% following 10 and 18 hours of incubation, respectively (Robinson et al., 1995).

From studies, it is apparent that the API20E system performance is comparable to the automated systems. It is safe to say that the API20E system is a robust bacteria identification method which can serve small and medium clinical microbiology laboratories that may not afford automated systems. Adhering to the manufacturer's instructions and good laboratory practice improves the performance of this method

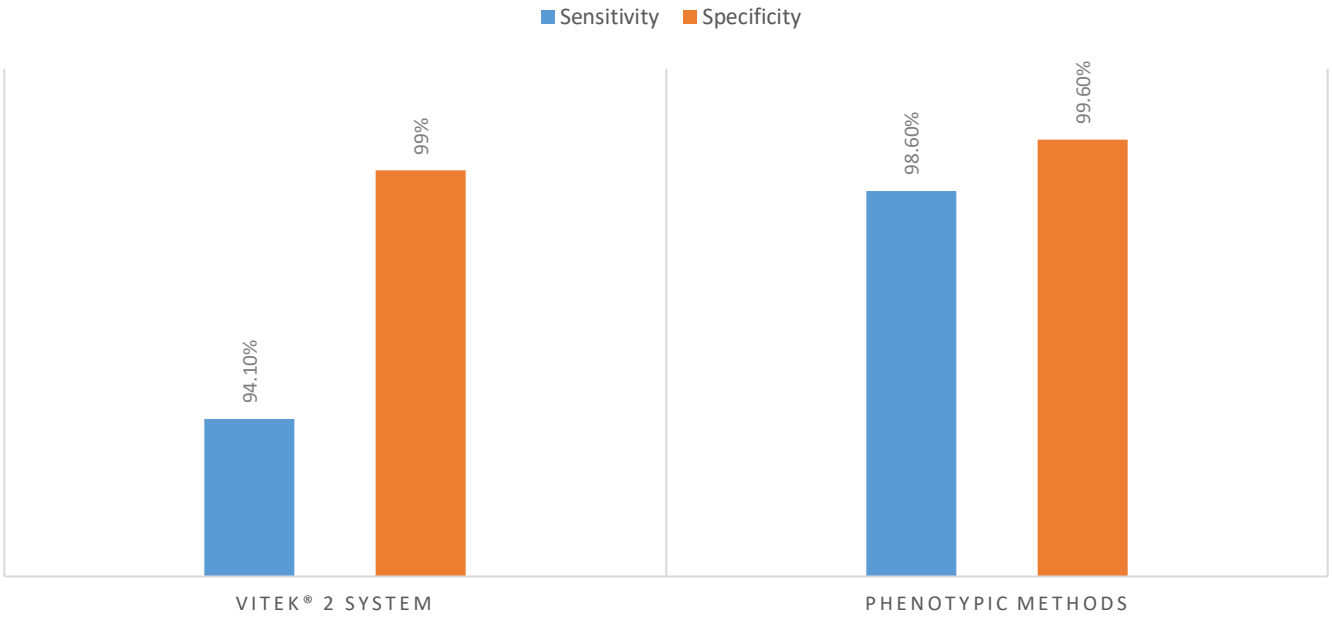
2.3 VITEK® 2 System for Identification

The VITEK® 2 system is an automated system for identification and Antimicrobial Susceptibility Testing (AST), which automatically performs all of the steps required for identification and AST after the preparation of a primary inoculum (Funke et al., 1998). This system facilitates kinetic analysis by reading each test every 15 min. The optical system combines multichannel fluorimeter and photometer readings to record fluorescence, turbidity, and colorimetric signals. The accuracy of the VITEK® 2 system in rapid detection and characterization of clinically significant gram-positive cocci was first evaluated by Bassel et al. (1997). The evaluation study revealed a 98.0% overall agreement with *Staphylococci, Streptococci,* and *Enterococci*, while there was a 1.7% and 0.3% rate misidentification and non-dentification of the isolates respectively. Additionally, there was a 96% accuracy in the identification of *S. aureus*; 97% accuracy for *S. epidermidis*; 97%

accuracy for *Staphylococcus haemolyticus*; 97% accuracy for *E. faecalis*; 91% for *E. faecium*; 95% for *S. agalactiae*; and 76% for *S. pneumoniae*.

A similar accuracy level was observed by Ligozzi et al. in identifying *Staphylococci*. In the study, the VITEK® 2 system failed to identify CoNS and *S. aureus*, which could be accounted for by the slow metabolism rate of some CoNS strains, leading to ambiguous results in the reaction wells. While Garcia- Garrote et al. reported an accuracy of 98.3% for the identification of *E. faecium*, a relatively low accuracy (71.4%) was observed by Ligozzi et al., and while in the latter study *E. faecum* was mostly misidentified as *E. casseliflavus-E. gallinarum*, in the former, most *E. faecium* isolates were misidentified as *E. hirae* or *E. durans*, and these discrepancies could not be resolved since simple tests are not available to discriminate among these species, whereas a simple motility test can discriminate *E. faecium* from *E. casseliflavus-E. gallinarum* (Garcia- Garrote et al., 2000; Ligozzi et al., 2002). Monteiro et al. in comparing the accuracy of the VITEK® 2 system with conventional culture-based and molecular (PCR) methods in the identification of 400 bloodstream bacteria pathogen, also observed a misidentification of *Enterococcus faecalis* strain by the VITEK® 2 system. Thus, the incorrect identification resulted in a 91.7% accuracy for the identification of *Enterococcus* spp compared with that observed by culture-based and molecular methods. A similar result with the findings of Ligozzi et al was also observed by Monteiro et al.- recording a 92.6 % accuracy in identifying Gram-positive cocci. An overall agreement of 98.7% was observed between the results obtained by phenotypic identification using conventional methods and the results of the genotypic methods (Monteiro et al., 2016). Figure 12 highlights the sensitivity and specificity of the VITEK® 2 system compared to the conventional phenotypic methods.

Average sensitivity and specificity of the conventional phenotypic methods and of the automated VITEK® 2 system in the Identification of Gram-positive cocci



The VITEK system has undergone several evaluations over the last two decades, leading to the improvement of the ID-GPC (Gram-positive cocci) identification card to the GP (Gram-positive cocci). This improvement has resulted in more efficiency for the system (Monteiro et al., 2016). A comparison of the old and new identification cards and found that the GP card correctly identified 235/249 (94.4 %) Gram-positive cocci, while the ID-GPC correctly identified 218/249 (87.5 %) isolates (Wallet et al., 2005). The main advantages of the VITEK automated system for species identification over conventional methods and antimicrobial susceptibility testing are speed and better workflow. There is still the need for improvements in their accuracy, and this would make them become more practical. In an analysis of vancomycin resistance in *Enterococcus sp*., the VITEK 2 system had a 100% accuracy (20/20 isolates) (d'Azevedo et al., 2009).

Table 5. Discrepant results between the automated VITEK® 2 system and Conventional methods of identification of Gram-positive cocci from blood culture

Conventional methods (Gram staining, culture, biochemical)	**Automated identification (VITEK® 2 system)**
Staphylococcus epidermidis	*Staphylococcus hominis*
Staphylococcus epidermidis	*Staphylococcus lentus*
Enterococcus faecalis	*Enterococcus gallinarum*
Staphylococcus haemolyticus	*Staphylococcus haemolyticus*
Staphylococcus epidermidis	*Staphylococcus capitis*

Monteiro et al., 2016

CHAPTER THREE
MATERIALS AND METHODOLOGY

3.1 Study Location:

The study was conducted at the Medical Microbiology Laboratory of the Lagos State University Teaching Hospital (LUTH), Idiaraba, Lagos State, a tertiary healthcare facility located in Ikeja, Lagos state, southwestern part of Nigeria. It is a 761-bed space hospital with special care for dental, pediatric, surgical and cancer patients. It also has other departments including internal medicine, obstetrics and gynecology, laboratory medicine, and allied services. It has both adult and pediatric intensive care units and burn unit for high-risk patients. The microbiology laboratory processes all types of specimens and has facilities for bacterial identification to species levels.

3.2 Study Population:

A total of 80 Gram-positive cocci that have been previously isolated from the Medical Microbiology laboratory of LUTH were studied, and identified from specimens including blood, urine or specimens from other normally sterile body sites, up to species level.

3.3 Sample processing:

This study used both conventional methods including Gram staining, catalase test, coagulase test, colony morphology and other methods including API and automated method VITEK for the characterization of Gram-positive cocci. Isolates were stored in glycerol broth at -80^{o}C prior to microbiological characterization. All isolated Gram-positive cocci were cultured on appropriate

culture media (Blood agar, nutrient agar, MacConkey agar), and the initial identification was based on colony morphology on the agar medium.

3.4 STAPHYLOCOCCUS SPECIES

3.4.1 Conventional methods for the identification of *Staphylococcus* species

- **Morphological identification of *Staphylococcus* species.**

Presumptive *Staphylococcus* spp. was initially subjected to morphological characterization including microscopy, Gram staining techniques to ascertain their shape and Gram status. Isolates that were positive or appeared blue or purple, with a coccoid colonial appearance under the microscope upon Gram-staining were selected for further tests.

Gram staining*:*

Thin smear of bacterial culture was made on clean glass slide, air dried and heat fixed. The smear was flooded with the primary Gram stain-crystal violet for 30 seconds. Slide were rinsed with distilled water and smear was flooded with Gram's iodine solution for 60 seconds. The slide was rinsed with distilled and subsequently with 95% ethyl alcohol and then distilled water again. The smear was flooded with safranin for 30 seconds and rinsed with distilled water and left to dry before observing under the microscope.

- **Biochemical identification of *Staphylococcus* spp.**

Isolates that appeared blue or purplish after Gram staining were subjected to both catalase and coagulase biochemical tests.

Catalase test: This was conducted using the Tube (slant) method with 1.0 ml of 3% H_2O_2 directly onto an 18- to 24-hour heavily inoculated pure culture grown on a nutrient agar slant as described by Reiner (2010). The culture was be observed for the appearance or absence of gas bubbles.

Coagulase test: Using glass slides, the slide test was used to detect bound coagulase, using a drop of physiological saline in which a portion of the isolated colony has been emulsified on two separate glass slides, and an addition of a drop of pooled human blood plasma to one of the suspensions, as described by Aryal (2018). Isolates for which clumping was observed within 10 seconds were regarded as coagulase positive. No plasma was added to the second suspension to differentiate any granular appearance of the organism from true coagulase clumping.

3.5 STREPTOCOCCUS SPECIES

3.5.1 Identification by Conventional methods

Conventional identification of streptococcal cultures was done by using colony morphology, catalase test, and hemolysis test. Aneja, (2003), APHA, (1998) and Bergey's Manual were consulted for proper interpretation of results and identification.

Colony morphology: Preliminary identification was done using the morphological characteristics of the culture colony including colony colour, size, margin, form, elevation and texture.

Catalase test**:** The catalase test is primarily used to differentiate between gram-positive cocci. Members of the genus *Staphylococcus* are catalase-positive, and members of the genera *Streptococcus* and *Enterococcus* are catalase-negative.

This was conducted using the Slide (drop) method, as described by Reiner (2010), with 1 drop of 3% H_2O_2 directly onto an 18- to 24-hour heavily inoculated pure culture grown on blood agar plate (BAP) at 35-37°C with ~5% CO_2 (or in a candle-jar). The suspension was be observed for the

appearance of vigorous bubbling on the glass slide. A *Staphylococcus* spp. strain was used as positive control and *S. pyogenes* was used as negative control.

The absence of bubbling from a transferred colony was regarded as indicative of a negative test while any bubbling from a transferred colony was taken as indicative of a positive test.

Hemolysis test: Hemolysis occurs around streptococcal colonies growing on BAP because of production of the cytolytic toxin streptolysin. Streptolysin causes transmembrane pore formation in red blood cells resulting in osmotic lysis. This test was used to identify *Streptococcus species*. Three patterns of hemolysis occur;

- α-Hemolysis: growth on blood plates causes incomplete destruction of blood cells. This produces dark green discoloration around bacterial colonies, reflecting the presence of biliverdin and other hemoglobin breakdown products.
- β-Hemolysis: growth on blood plates causes complete destruction of blood cells, resulting in transparency of the region surrounding bacterial colonies.
- γ-Hemolysis: no observable destruction of blood cells surrounding bacterial colonies.

3.5.2 Preparation of API strip

- An incubation box (tray and lid) will be prepared and about 5ml of distilled water will be distribute into the honey-combed wells of the tray to create a humid atmosphere.
- The strain reference on the elongated flap of the tray will be record
- The strip will be removed from its individual packaging.
- The strip is placed in the incubation box.

Preparation of the inoculum

- An ampule of API Suspension Medium (2ml) will be opened as indicated in the user guide.

- Using a swab, all the culture from the previously prepared subculture plate will be harvested.
- A dense suspension with a turbidity greater than 4 McFarland will then be prepared, which will be used immediately after preparation.

Inoculation of the strip

- In the first half of the strip (tests VP to ADH), the prepared suspension will be distributed, avoiding the formation of bubbles.

  - For the tests VP to LAP: approximately 100 µl of the suspension will be distributed into each cupule.

  - For the ADH test: only the tube will be filled
- In the second half of the strip (tests RIB to GLYG): The rest of the suspension (appr. 0.5ml) is transferred into an open ampule of API GP Medium as indicated in the user guide. Mix well.

  - The new suspension will then be distributed into the tubes only.
- The cupule of the tests ADH to GLYG will be filled with mineral oil to form a convex meniscus.
- The lid will be placed on the tray.
- It will be incubated at 36°C ± 2°C in aerobic conditions for 4 - 4½ hours to obtain a first reading and for 24 hours (± 2 hours) to obtain a second reading if required.

Reading the strip

- After 4 hours of incubation:

Add the reagents:

- VP test: 1 drop of each of VP 1 and VP 2.

- HIP test: 2 drops of NIN.

- PYRA, αGAL, ßGUR, ßGAL, PAL and LAP tests: 1 drop of each of ZYM A and ZYM B After 10 minutes, the reactions are read by referring to the Reading Table.

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS			
				NEGATIVE		POSITIVE	
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / wait 10 min (3)			
				Colorless		Pink-Red	
HIP	hippuric acid	0.4	hydrolysis (HIPpuric acid)	NIN / wait 10 min			
				Colorless/Pale blue Bluish-grey		Dark blue/Violet	
ESC	esculin ferric citrate	1.16 0.152	ß-glucosidase hydrolysis (ESCulin)	4 hrs.	24 hrs.	4 hrs.	24 hrs.
				Colorless Pale yellow	Colorless Pale yellow Light grey	Black Grey	Black
PYRA	pyroglutamic acid-ß-naphthylamide	0.0256	PYRrolidonyl Arylamidase	ZYM A + ZYM B / 10 min (PYRA to LAP) (1) if necessary, decolorize with intense light			
				Colorless or very pale orange		Orange	
αGAL	6-bromo-2-naphthyl-αD-galactopyranoside	0.0376	α-GALactosidase	Colorless		Violet	
ßGUR	naphthol ASBI-glucuronic acid	0.0537	ß-GlUcuRonidase	Colorless		Blue	
ßGAL	2-naphthyl-ßD-galactopyranoside	0.0306	ß-GALactosidase	Colorless or Very pale violet		Violet	
PAL	2-naphthyl phosphate	0.0244	ALkaline Phosphatase	Colorless or Very pale violet		Violet	
LAP	L-leucine-ß-naphthylamide	0.0256	Leucine AminoPeptidase	Colorless		Orange	
ADH	L-arginine	1.9	Arginine DiHydrolase	Yellow		Red	
				4 hrs.	24 hrs.	4 hrs.	24 hrs.
RIB	D-ribose	1.4	acidification (RIBose)	Red	Orange/ Red	Orange/ Yellow	Yellow
ARA	L-arabinose	1.4	acidification (ARAbinose)	Red	Orange/ Red	Orange/ Yellow	Yellow
MAN	D-mannitol	1.36	acidification (MANnitol)	Red	Orange/ Red	Orange/ Yellow	Yellow
SOR	D-sorbitol	1.36	acidification (SORbitol)	Red	Orange/ Red	Orange/ Yellow	Yellow
LAC	D-lactose (bovine origin)	1.4	acidification (LACtose)	Red	Orange/ Red	Orange/ Yellow	Yellow
TRE	D-trehalose	1.32	acidification (TREhalose)	Red	Orange/ Red	Orange/ Yellow	Yellow
INU	inulin	5.12	acidification (INUlin)	Red	Orange/ Red	Orange/ Yellow	Yellow
RAF	D-raffinose	3.12	acidification (RAFfinose)	Red	Orange/ Red	Orange/ Yellow	Yellow
AMD	starch (2)	2.56	acidification (AmiDon)	Red	Orange/ Red	Orange/ Yellow	Yellow
GLYG	glycogen	1.28	acidification (GLYcoGen)	Red or Orange		Bright yellow	

Figure 8 API 20 READING TABLE

3.6 Identification and Antibiotics Sensitivity Testing Using VITEK

Principle

The Vitek-2 Compact (30 card capacity) system uses a fluorogenic methodology for organism identification and a turbidimetric method for susceptibility testing using a 64 well card that is barcoded with information on card type, expiration date, lot number and unique card identification number. Test kits available include ID-GN (gram negative bacillus identification), ID-GP (gram positive cocci identification), AST-GN (gram negative susceptibility) and AST-GP (gram positive susceptibility). The Vitek-2 ID-GN card identifies 154 species of Enterobacteriaceae and a select group of glucose non-fermenting Gram-negative organisms within 10 hours. The Vitek-2 ID-GP card identifies 124 species of staphylococci, streptococci, enterococci and a select group of Gram-positive organisms within 8 hours or less. The Vitek-2 Antimicrobial Susceptibility Tests (AST) is for most clinically significant *Staphylococcus spp*., *Enterococcus spp*., and *Streptococcus agalactiae* as well as aerobic Gram-negative bacilli. Using this technique provides susceptibility results in less than 18 hours (Sanders, 2019).

Procedure

- An inoculum will be prepared from a pure culture, according to good laboratory practices. In case of a mixed culture, an isolation step will be required. A purity check plate will be used to ensure that a pure culture was used for testing.
- 3.0 ml of sterile saline will be transferred aseptically (0.45% to 0.5% NaCl, pH 4.5 to 7.0) into a clear plastic (polystyrene) test tube (12mm x 75mm).
- A sterile swab will be used to transfer enough morphologically similar colonies to the saline tube prepared in step 2. A homogenous organism suspension will be prepared with

a density equivalent to the appropriate McFarland standard using the VITEK-2 DensiCHEK Plus.

- In a second tube containing 3.0ml of saline, 145µl of the suspension prepared in step 3 will be transferred for AST-GN cards, this tube will then be placed in the cassette with a susceptibility card. The tube with the initial bacterial suspension will also be used for inoculation of an identification card.

3.7 DATA ANALYSIS

Comparative assessment data are presented in the form of tables and charts using percentages of the total population. STATA 3.1 software and EPIDAT 8.0 was used to analyze the extracted data. There were four possibilities for analysis of the identification results: (i) correct identification, in which strains were correctly identified to the species level or strains with low discrimination are resolved (by simple additional tests); (ii) low discrimination, in which strains with low discrimination are not resolved (and cannot be resolved by simple additional tests); (iii) misidentification, in which discrepant results are obtained for strains and (iv) no identification, in which no identification was provided. The mean time for result generation was also calculated for all identifications.

The accuracy, specificity, sensitivity and predictive values of the techniques were determined as follows.

$$\textbf{Sensitivity} = \frac{\text{True positive}}{\text{True positive+ False negative}}$$

$$\textbf{Specificity} = \frac{\text{True negative}}{\text{True negative + False positive}}$$

$$\textbf{Positive predictive value (PPV)} = \frac{\text{True positive}}{}$$

True positive + False positive

Negative predictive value (NPV) = $\frac{\text{True negative}}{\text{True negative + False negative}}$

Accuracy = $\frac{\text{True positives + True negatives}}{\text{True positive + True negatives + False positives + False negatives}}$

CHAPTER FOUR

RESULTS

Gram positive cocci clinical isolates (n = 80) were evaluated (Table 1). All isolates were identified by conventional identification methods, VITEK, and API methods described by Devriese et al. and Kloos and Schleifer. The Vitek GPI card and API system were used according to the instructions of the manufacturers. In evaluating the sensitivity, specificity, PPV and NPV of the conventional methods and VITEK methods, the API method was used as gold standard in this study.

Agreement of the Vitek card system with API was 51.25% (Table 2). Twelve Staphylococcus isolates were identified incorrectly as either *Micrococcus, Kocuria, Enterococcus, Gamella, Aerococcus*. Strains of *S. hominis, S. chromogenes, S. sciuri, S. xylosus*, and *S. epidermidis* were misidentified as *S. aureus.* Approximately 55.5% of all *S. hominis*, 60% of all *E. faecium*, and 80% of all *S. xylosus* were misidentified by the VITEK method. The accuracy of the VITEK system among species varied from 0% for *S. epidermidis, S. caprae*, and *Streptococcus porcinus* to 100% for *E. faecalis* (Table 3). The VITEK system identified incorrectly one *S. epidermidis, S. chromogenes, and S. hominis* as *Micrococcus luteus*. Except for *S. aureus* (100%), the conventional method was in agreement with API less than 60% of the time. Twenty percent (20%) of the isolates identified by the conventional method were identified as CoNS, with no specific species/strain named while a total of 12.5% were identified as CoPS. All the isolates merely identified as either CoNS or CoPS by the conventional method were identified to the species level by the VITEK method as *S. aureus, Kocuria rosea, S. hominis, S. haemolyticus, S. lentus, Micrococcus luteus, Kocuria varians, Kocuria kristinae*, and *Gamella sanguinis*. While the VITEK method misidentified the only Streptococcus species identified by the API standard, the

conventional method accurately identified it as *Streptococcus porcinus* in agreement with the API method. Meanwhile, the conventional method had a 0% agreement with the API method in identifying the Enterococcus isolates.

Table 6 Number of Gram-positive cocci identified at various probabilities of identification by API

Species (no of strains)[a]	**Probability of identification (no. of strains)**				
API					
	90-99	80-89	70-79	60-69	< 60
S. hominis (11)	0	3	1	7	0
S. haemolyticus (8)	6	0	2	0	0
S. aureus (28)	21	6	1	0	0
S. capitis (2)	0	0	0	0	2
S. chromogenes (2)	2	0	0	0	0
S. sciuri (1)	0	0	1	0	0
S. lentus (7)	6	1	0	0	0
S. xylosus (5)	5	0	0	0	0
S. epidermidis (6)	2	0	2	1	1
S. caprae (1)	0	0	0	0	1
Streptococcus					
S. porcinus (1)	0	1	0	0	0
Enterococcus					
E. faecium (5)	5	0	0	0	0
E. faecalis (3)	0	0	0	0	3

[a] Strains identified with API

Table 7 Number of Gram-positive cocci identified at various probabilities of identification by VITEK

Species Identified (no of strains)[a]	**Probability of identification (no. of strains)**			
	Vitek GPI Card			
	90-99	80-89	70-79	60-69
***S. hominis* (6)**	5	1	0	0
***S. haemolyticus* (11)**	11	0	0	0
***S. aureus* (26)**	23	3	0	0
***S. sciuri* (1)**	1	0	0	0
***S. lentus* (12)**	9	3	0	0
***S. xylosus* (1)**	1		0	0
***Staphylococcus kloosii* (1)**	NA	NA	NA	NA
Enterococcus				
***E. faecium* (1)**	NA	NA	NA	NA
***E. faecalis* (6)**	5	1	0	0
***Enterococcus gallinarum* (2)**	0	2	0	0
***Enterococcus casseliflavus* (1)**	0	1	0	0
Others				
***Micrococcus luteus* (3)**	3	0	0	0
***Micrococcus lylae* (1)**	1	0	0	
***Gemella sanguinis* (1)**	0	1	0	0
***Kocuria kristinae* (3)**	3	0	0	0
***Kocuria varians* (2)**	0	1	0	0
***Aerococcus viridians* (1)**	0	1	0	0
***Kucuria rosea* (1)**	1	0	0	0

[a] Strains identified with API

Table 8 Identification of Gram-positive cocci by VITEK compared with API

Species (no of strains)[a]	**No. (%) with correct identification**	**Incorrect species identification (no., and species)**
Staphylococcus		
***S. hominis* (11)**	5(45.5%)	1, *Kucuria rosea* 1, *Micrococcus lylae* 1, *Staphylococcus aureus* 1, *Micrococcus luteus* 1, *Aerococcus viridians* 1, *Gemella sanguinis*
***S. haemolyticus* (8)**	5(62.5%)	1, *S. hominis* 2, *S. lentus*
***S. aureus* (28)**	20(71%)	1, *S. sciuri* 1, *S. lentus* 3, *S. haemolyticus* 2, *Kocuria kristinae* 1, *Kocuria varians*
***S. capitis* (2)**	0(0)	1, *S. haemolyticus* 1, *S. kloosii*
***S. chromogenes* (2)**	0(0)	1, *S. aureus* 1, *Micrococcus luteus*
***S. sciuri* (1)**	0(0)	1, *S. aureus*
***S. lentus* (7)**	5(71%)	1, *S. intermedius* 1, *Kocuria kristinae*
***S. xylosus* (5)**	1(20%)	1, *E. faecalis* 1, *S lentus* 1, *S. aureus* 1, *S. haemolyticus*
***S. epidermidis* (6)**	0(0)	2, *S. aureus* 3, *S. lentus* 1, *Micrococcus luteus*

***S. caprae* (1)**	0(0)	1, *S. haemolyticus*
Streptococcus		
***S. porcinus* (1)**	0(0)	1, *E. faecalis*
Enterococcus		
***E. faecium* (5)**	2(40%)	1, *E. casseliflavus*
		2, *E. gallinarum*
***E. faecalis* (3)**	3(100%)	
Total (80)	**41 (51.25%)**	**39 (48.75%)**

[a] Strains identified with API

The probability of identification of the correctly identified isolates by the VITEK and API methods is shown in Table 4 and 5. Most isolates (55 of 80) were identified with a ≥90% level of confidence by the VITEK method. The probability of identification varied considerably for strains identified by the API system. Only 1strain was identified with an excellent level of identification by the API system while 47 out of 80 (58.75%) were identified with ≥90% level of confidence. Table 6 shows the sensitivity, specificity, positive predictive value and negative predictive value of both the conventional and VITEK methods of identification.

Table 9 Number of Gram-positive cocci identified at various probabilities of identification by VITEK

Species Identified (no of strains)[a]	**Probability of identification (no. of strains)**			
	Vitek GPI Card			
	90-99	80-89	70-79	60-69
***S. hominis* (6)**	5	1	0	0
***S. haemolyticus* (11)**	11	0	0	0
***S. aureus* (26)**	23	3	0	0
***S. sciuri* (1)**	1	0	0	0
***S. lentus* (12)**	9	3	0	0
***S. xylosus* (1)**	1		0	0
***Staphylococcus kloosii* (1)**	NA	NA	NA	NA
Enterococcus				
***E. faecium* (1)**	NA	NA	NA	NA
***E. faecalis* (6)**	5	1	0	0
***Enterococcus gallinarum* (2)**	0	2	0	0
***Enterococcus casseliflavus* (1)**	0	1	0	0
Others				
***Micrococcus luteus* (3)**	3	0	0	0
***Micrococcus lylae* (1)**	1	0	0	
***Gemella sanguinis* (1)**	0	1	0	0
***Kocuria kristinae* (3)**	3	0	0	0
***Kocuria varians* (2)**	0	1	0	0
***Aerococcus viridians* (1)**	0	1	0	0
***Kucuria rosea* (1)**	1	0	0	0

[a] Strains identified with VITEK

Table 10 Number of Gram-positive cocci identified at various probabilities of identification by API

Species (no of strains)[a]	**Probability of identification (no. of strains)**				
API					
	90-99	80-89	70-79	60-69	< 60
S. hominis (11)	0	3	1	7	0
***S. haemolyticus* (8)**	6	0	2	0	0
***S. aureus* (28)**	21	6	1	0	0
***S. capitis* (2)**	0	0	0	0	2
***S. chromogenes* (2)**	2	0	0	0	0
***S. sciuri* (1)**	0	0	1	0	0
***S. lentus* (7)**	6	1	0	0	0
***S. xylosus* (5)**	5	0	0	0	0
***S. epidermidis* (6)**	2	0	2	1	1
***S. caprae* (1)**	0	0	0	0	1
Streptococcus					
***S. porcinus* (1)**	0	1	0	0	0
Enterococcus					
***E. faecium* (5)**	5	0	0	0	0
***E. faecalis* (3)**	0	0	0	0	3

[a] Strains identified with API

Table 11 Sensitivity, specificity, PPV and NPV of conventional and VITEK methods

Gram + Cocci Group	**Conventional Method**				**VITEK**			
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Staphylococcus	94.3	0	100	0	83	43	83	43
Streptococcus	100	0	100	0	0	0	0	0
Enterococcus	0	0	0	0	62.5	0	100	0

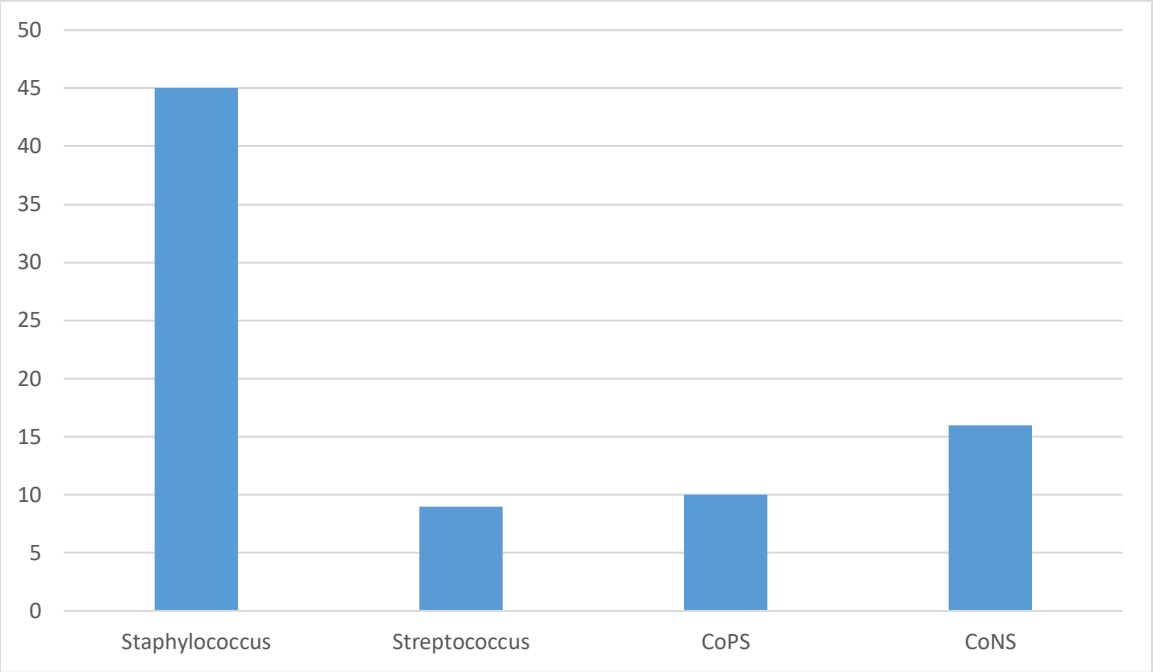


Figure 9 Species Identification by Conventional method

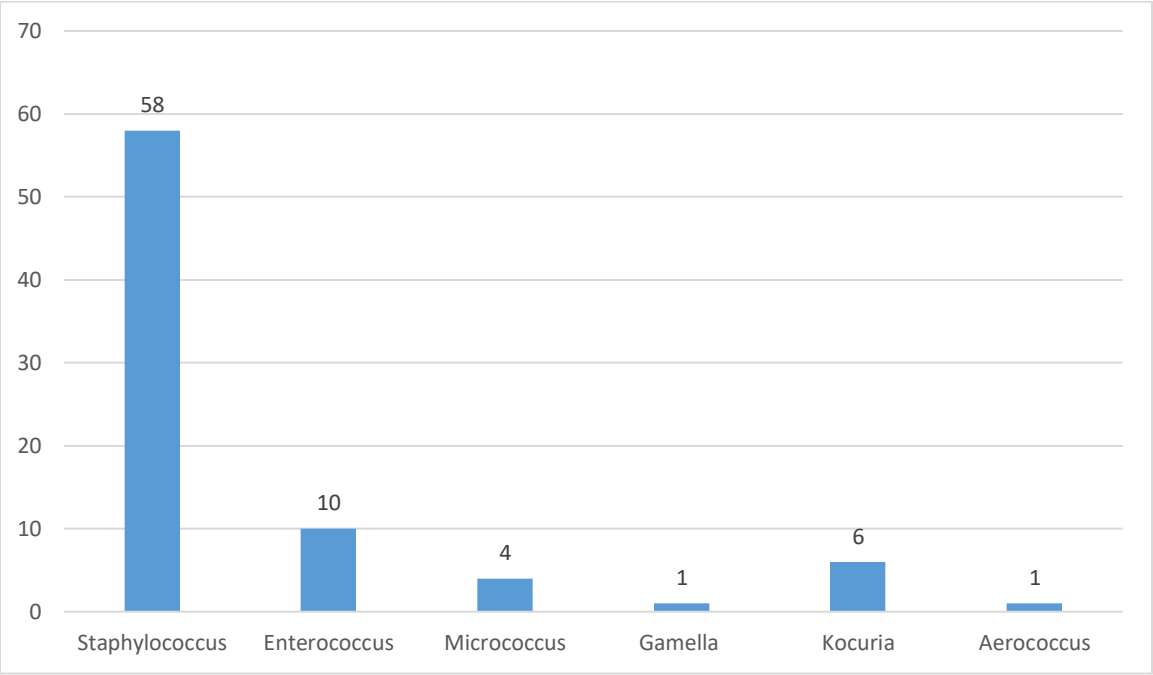


Figure 10 Species identification by VITEK

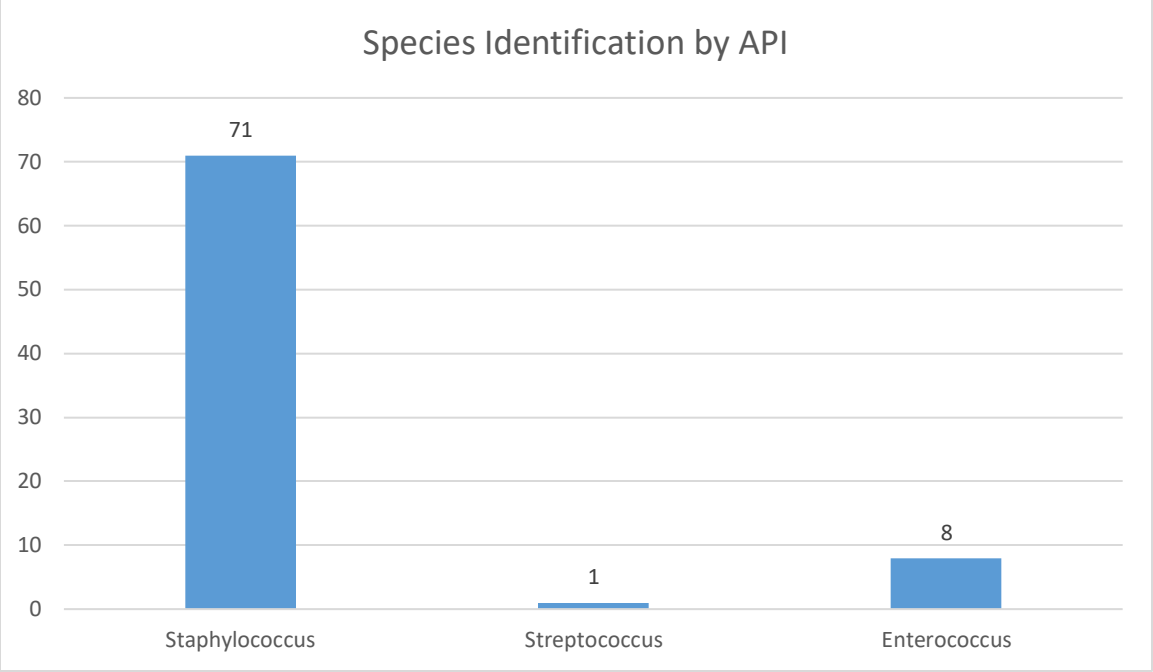


Figure 11 Species Identification by API

CHAPTER FIVE

DISCUSSION AND CONCLUSION

This prospective study was performed under routine diagnostic conditions. A collection of clinically relevant strains (n=80) of Gram-positive cocci isolated in the diagnostic laboratory was investigated. Accurate identification of these strains, mostly obtained from normally sterile body sites, was attempted with the commercially available API system which was used as the gold standard. VITEK identification and Conventional identification using colony morphology, Gram reaction, biochemical tests and hemolysis tests were performed in parallel.

It was demonstrated in this study that VITEK method of identification has an overall improved ability to identify gram-positive cocci compared to that of the conventional in the sense that all isolates were identified to the species level by the VITEK system while approximately 32.5% of the isolates could not be identified even to the genus level by the conventional system. 100% of the isolate that could not be discriminated at any taxonomic level biochemically (26 of 80) were assigned to a species by the VITEK and API methods.

VITEK identification yielded discrepant results for 16 of the 80 strains which were assigned to the species level by the API 20 Strep system. For the isolates with discrepant results, it was concluded that VITEK method had a lower ability to correctly identify the isolates (or at least had less discriminative power. The results of the conventional identification helped to prove that the conventional method had the least discriminative power among the three methods owing to fact that a large percentage of the isolates could not be assigned a species whereas they were identified to species level by both the API and VITEK systems.

The VITEK method demonstrated a high percentage of correct identification for *S. aureus* (71%) and *S. lentus* (71%). However, the method had a zero percent identification for *S. epidermidis,* another common human pathogen as *S. aureus.* These results partly agree with the percent correct identification found by Almeida et al. (1) and Ruoff et al. (9). When these results are compared with that of the conventional method, a slight increase in correct identification is observed, with the conventional method recording a 100% correct identification for *S. aureus* and 33.3% identification for *S. epidermidis*. Therefore, the frequency of a particular Staphylococcus species in an isolate set may affect overall percent correct identification between rapid test systems and conventional methods.

There was a relatively low rate of correct identifications observed in our study with *E. faecium* for both the VITEK and Conventional methods. Garcia Garrote et al. reported the accuracy of the VITEK 2 system to be 98.3 and 76.3% in the identification of *E. faecalis* and *E. faecium*, respectively; in that study the *E. faecium* was mostly misidentified as *E. casseliflavus*-*E. gallinarum*, which is also the case in this present study. However, both *E. faecalis* and *E. faecium* were misidentified as streptococcus by the conventional method. We have no explanations for accuracy shown by the conventional method in our study and for the prevalence of misidentification with streptococcus. Nevertheless, in the clinical setting, reasons for species identification of enterococci are very limited (serious infections, such as endocarditis, or epidemiological surveillance within hospitals). Several taxonomy changes have been introduced in the Enterococcus genus, mainly involving species other than *E. faecalis*. The difficulties of commercial tests in the identification of species other than *E. faecalis* probably reflects problems with the new species definition or in the criteria available for identifying them.

In conclusion, this study provides a mixed result for the validation of both the VITEK method and conventional method in identifying gram positive cocci in the laboratory, in that while the conventional method had a near average accuracy of 55%, it could not identify to species level about 35% of the isolates which is not satisfactory in our opinion. Also, while the VITEK system had high sensitivity for one or more of the gram-positive cocci groups, the overall accuracy and agreement with the standard is relatively low and not satisfactory. While we are unable to ascertain the reason for this, it is recommended that further evaluation is required.

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