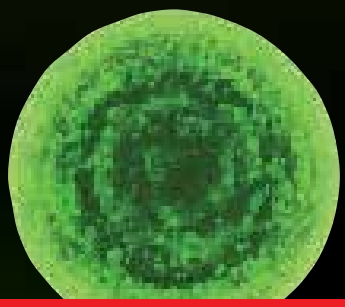

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Staphylococcus Aureus

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Meet the editors

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Contents

Preface

The *Staphylococcus aureus* strain is the most important strain in the Staphylococcus genus. It has colonized the skin and mucosal surface of the human body. However, Methicillin-Resistant *Staphylococcus aureus* (MRSA) has emerged as a frightening human pathogen and has been a leading cause of hospital and community-acquired infections over the past several decades. MRSA has become a well-known etiologic agent of a wide variety of diseases, including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis, and post-surgical toxic shock syndrome, with substantial rates of morbidity and mortality. One of the reasons for the progress of this human pathogen is its great variability, occurring at different periods and places with diverse clonal types and antibiotic resistance patterns within regions and countries. Although infections can cause serious problems in the general population, such infections can be particularly severe for children, the elderly, and immunocompromised patients.

Antimicrobial resistance among nosocomial pathogens is a significant problem in many countries, with serious consequences including increased medical costs and high rates of morbidity and mortality.

Clindamycin is a conventional treatment for soft tissue infection caused by MRSA. However, treatment failure should be taken into consideration, especially with some antibiotics such as vancomycin.

Patients with MRSA bacteremia have reported increased morbidity, longer length of hospital stay, and higher costs when compared to patients with MSSA (Methicillin-Susceptible *Staphylococcus aureus*) bacteremia. At this point, MRSA has spread from the hospitals into communities, infecting individuals with no known risk factors. Measures to control the emergence and spread of MRSA are justified because there are fewer options available for the treatment of MRSA infections and because these strains spread amongst people who have a weak and compromised immune system and are hence more susceptible to infections. Recently, it has also spread among ordinary healthy people in the community.

MRSA strains are harboring resistant genes to several antibiotics including methicillin, aminoglycosides, and others. Recently, there have been several reports related to the failure of treatment plans caused by MRSA that lead to Vancomycin Intermediate *Staphylococcus aureus* strains (VISA) or, in sporadic cases, resistance to the drug of choice.

This book highlights new areas for the treatment of MRSA using natural products. The implementation of specific products produced by this organism can help scientists to obtain a new window for treatments such as anticancer chemotherapy, antioxidant, etc.

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Chapter 1

Non-microbial Natural Products That Inhibit Drug-Resistant *Staphylococcus aureus*

Jactty Chew, Suat-Cheng Peh and Teow Sin Yeang

Additional information is available at the end of the chapter

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Abstract

Drug resistance developed in human pathogenic bacteria is emerging and has become a global problem. Methicillin-resistant *Staphylococcus aureus* (MRSA) spreading in both hospital and community areas has posed a great impact to global public health. Current antibiotics used against these resistant strains are no longer efficacious and the search for new alternative is in urgent need. In the past decades, natural products have demonstrated multiple biological activities in biomedical areas including their antibacterial actions against various drug-resistant bacteria. More promisingly, some natural products could reverse the resistance of bacteria to the antibiotics, making the target bacteria susceptible to these drugs again. Numerous natural products have also exhibited potent synergism against the drug-resistant bacteria when used in combination with various types of antibiotics. Recently, several antibacterials derived from microbes have been developed and approved by Food and Drug Administration (FDA) for clinical use. In this chapter, we discuss the potential use of non-microbial natural products in controlling *Staphylococcus aureus* (*S. aureus*)'s growth, and the underlying challenges in developing the natural products into clinical applications.

Keywords: natural products, *Staphylococcus aureus*, methicillin-resistant, antibacterial, MRSA

1. Introduction

Staphylococcus aureus (S. aureus) is a coagulase-positive Gram-positive cocci bacterium, commonly found on human skin and mucous membranes. Up to 30% of the world population is colonised by this bacterium [1]. Despite being part of the human normal microbiota, it is known to be a pathogen causing various levels of diseases ranging from mild skin infections

such as boils and rashes, to life-threatening diseases such as persistent bacteraemia, sepsis, and pneumonia [2]. The pathogenicity of this bacterium is attributed to its vast arrays of virulence factors such as adhesins, production of enzymes and toxins, biofilm formation, and evasion of immunity strategies [3–5]. Apart from the known virulence factors, this opportunistic pathogen is best known for its formidable reputation due to its antibiotic-resistant phenotype. Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) are among the two recognised health threats to the humans. As of now, MRSA is listed as a 'serious threat' by Centres for Disease Control and Prevent (CDC) and 'priority pathogen' by the World Health Organisation (WHO), while VRSA is listed as 'concerning threat' by CDC.

Despite the rapid advancement of modern medicine, *S. aureus* infections remain highly prevalent in the human populations as transmission of these pathogens can occur through direct contact [6, 7]. Drug-resistant *S. aureus* particularly MRSA can be defined either as healthcare- or community- associated, based on the '48-hour rule'. In the former, MRSA infections develop after 48 hours from hospital admission while the later develops within 48 hours of admission. In this classification system, there are three categories of MRSA infections, namely (i) healthcare-associated, hospital-onset, (ii) healthcare-associated, community onset and (iii) community-associated MRSA infections. Essentially, healthcare-acquired MRSA infections (HA-SA or HA-MRSA) lead to bacteraemia, infective endocarditis, and prosthetic-associated infections while community-acquired MRSA infections (CA-MRSA) often lead to skin and soft tissues infection as well as community-acquired pneumonia in healthy individuals [2, 8, 9]. Compounding to the situation, MRSA has been reported to infect livestock including pigs, poultry, and cattle. Livestock-associated MRSA (LA-MRSA) can be transmitted to individuals handling these infected livestock [10]. In the early days, *S. aureus* has already been recognised as the main culprit causing hospital-acquired infections (HAIs) such as surgical site infections, bloodstream infections, and pneumonia [11]. The epidemiology of *S. aureus* shifted in the 2000s with the observation of MRSA infections dominating HAIs. In fact, MRSA strains account for up to 75% of all *S. aureus* infections in different part of the world [12–17]. In the US, for instance, MRSA causes approximately 11,000 deaths annually [18].

Antibiotic-resistant *S. aureus* is known to be associated with higher morbidity and mortality rates as compared to antibiotic-susceptible strains [19–21]. In the last decade, studies show that MRSA alone causes more death in the US hospitals than of HIV/AIDS, viral hepatitis, and tuberculosis in combination [22, 23]. In addition to health burden, these antibiotic-resistant *S. aureus* also imposes economic burden in order to eliminate the associated infections [24, 25]. The bacterium develops resistance to nearly all antibiotics introduced to treat infections caused by the bacterium. In 2011, the Expert Panel of the Infectious Diseases Society of America (IDSA) presented an evidence-based guideline for the management of antibiotic-resistant *S. aureus* infections, including antibiotic choices in both adult and paediatric patients [26]. The key antibiotic choices are described below.

1.1. Vancomycin

Vancomycin, a glycopeptide, was first introduced in the 1960s and has been the most reliable therapeutic agent for MRSA infections, including bacteraemia and endocarditis [27]. This broad spectrum antibiotic is a cell wall synthesis inhibitor. It binds to the c-terminal

of D-Ala-D-Ala residues of the peptides of the N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) murein subunits, preventing transpeptidases from forming the peptide bridge between peptidoglycan chain, leading to bacterial cell death. However, there is also a group of *S. aureus* resistant to vancomycin known as VRSA.

1.2. Daptomycin

This antibiotic is a promising alternative to vancomycin for infections caused by MRSA. This cyclic lipopeptide was approved for clinical use in the U.S in 2003 and Europe in 2006. Daptomycin targets only Gram-positive bacteria and is commonly used for complicated skin and skin-structure infections, bacteraemia, and right-sided endocarditis [27]. This antibiotic, however, is not recommended for the pneumonia caused by MRSA. Some studies indicated that daptomycin interacts with pulmonary surfactants present in the lung tissues, leading to the inhibition of daptomycin antibacterial activity [2, 28]. Daptomycin works by targeting the cytoplasmic membrane of bacteria in a calcium ion-dependent manner. In the presence of calcium ions, daptomycin aggregates and forms micellar structures. Daptomycin is then inserted into the membrane and binds strongly to phosphatidylglycerol headgroups leading to depolarisation and permeabilisation of the membrane. This then leads to cytoplasmic content leakage and cell death [29, 30]. The emergence of daptomycin-resistant *S. aureus* is relatively uncommon. However, increasing records of daptomycin-resistant *S. aureus* have been reported [31–33].

1.3. Linezolid

Linezolid, an oxazolidinone, was first approved by the FDA in 2000 for skin bacteraemia and pneumonia-origin *S. aureus* infections [1]. Linezolid is considered as a standard broad-spectrum intravenous therapies directed towards vancomycin- and teicoplanin-resistant Gram-positive pathogens, including MRSA. Linezolid inhibits protein synthesis by binding to the 23S subunit of the 50S ribosome. Linezolid-resistant *S. aureus* is relatively uncommon. Resistant strains have been previously reported in staphylococci involving mutations in the 23S rRNA and rRNA methyltransferase. These mutations prevent the binding of linezolid to the ribosome for interfering protein synthesis [34].

1.4. Ceftaroline

Ceftaroline is a fifth generation cephalosporin with a broad-spectrum bactericidal activity against both Gram-positive bacteria including MRSA and some Gram-negative bacteria. This antibiotic is used primarily for the treatment of acute bacterial skin and skin structure infections, and community-acquired bacterial pneumonia caused by *S. aureus* [35]. Ceftaroline has an enhanced affinity for penicillin binding protein 2a (PBP2a), thus is an ideal antibiotic choice for MRSA infections. This antibiotic is relatively new, and was approved for use in 2010 in the U.S, 2012 in Europe, and 2013 in Australia [36]. However, the emergence of ceftaroline resistance in different parts of the world with a demonstrated decrease of PBP2a binding affinity and heteroresistance, has been documented [37–39]. The associated mechanisms of resistance involve glutamic acid-to-lysine substitutions in the non-penicillin binding domain and the transpeptidase domain of the PBP2a [39, 40].

There is increasing evidence demonstrating that *S. aureus* is becoming resistant against all possible antibiotic choices used to treat the infections in the past. Hence, the search for and development of new antibacterials against drug-resistant *S. aureus* is of pivotal importance. Natural products represent an enormous reservoir of compounds that are diverse in structures and chemical properties. These compounds have been used as antibiotics, such as penicillin and streptomycin. The discovery and use of natural products as antibiotics led to the Golden Age of antibiotics in the 1950s to 1960s. In the past decades, many pharmaceutical companies moved away from natural products programmes partly due to a shift to both high-throughput screening and combinatorial synthesis that focus on small synthetic molecules [41, 42]. However, these approaches are proven to have limited successes [43]. In 2015, the Nobel Prize in Physiology or Medicine was awarded to William C. Campbell and Satoshi Omura, and Youyou Tu for their discovery of new anti-parasitic drugs of natural sources, Avermectin and Artemisinin, respectively. This marks the new milestone and brings optimism for natural product drug discovery. Antimicrobial properties of countless natural products have been tested on *S. aureus* and an earlier review summarises these research findings collected between 1995 and 2003 [44]. The purpose of this review is to provide an update on natural products that have been shown to demonstrate promising bactericidal effects against drug-resistant *S. aureus*, published in journal between 2014 and 2017. The resistance mechanisms of drug-resistant *S. aureus* will be discussed, followed by new anti- *S. aureus* agents collected from non-microbial natural products, their potential synergism with antibiotics, the molecular targets and mechanisms of these agents, and potential challenges in developing them into clinical trials.

2. Mechanism of *S. aureus* antimicrobial resistance

Generally, bacteria acquire resistance against antibiotics via different molecular mechanisms, including enzymatic inactivation of antibiotics, alteration of antibiotics target(s) leading to decreased affinity for the antibiotics, removing antibiotics via efflux pumps and changing membrane permeability [45, 46]. *S. aureus* is known to resist all the clinically approved antibiotics using various resistance mechanisms mentioned above. The detailed resistance mechanisms for important antibiotics, including penicillin, methicillin, and vancomycin are discussed below.

2.1. Penicillin resistance

Penicillin was first isolated from a soil fungus, *Penicillium* in the 1940s. This antibiotic was once thought to be a miracle drug as it could cure previously fatal infections. However, few years after its introduction, penicillin resistance including penicillin-resistant *S. aureus* was isolated from hospitals. Penicillin resistance of *S. aureus* is highly prevalent with up to 86% of clinical *S. aureus* isolates being resistant to the antibiotic in the US [47]. Meantime, far way in Australia, a similar observation was made as 80% of *S. aureus* isolates were resistant to penicillin [48]. Penicillin resistance in staphylococci is mediated by the production of enzyme penicillinase or beta-lactamase encoded by the *blaZ* gene. This enzyme inactivates the antibiotic by hydrolysis of the beta-lactam ring of the antibiotic [49]. Studies show that penicillinase genes can be present on either plasmid of the chromosome of *S. aureus* [50].

2.2. Methicillin resistance

Methicillin is a penicillinase-resistant beta-lactam. It was first introduced in 1950s and prescribed for *S. aureus* infection. The first MRSA was documented in 1961 in the UK while the first MRSA in the US was first reported in 1968. Since then, many MRSA clones spread to every corner of the globe. Methicillin resistance is usually encoded by *mecA* gene that is located in a mobile genetic element of *S. aureus*, known as the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*). *mecA* is responsible for the synthesis of low-affinity PBP2a which leads to decreased methicillin binding. Methicillin resistance confers broad spectrum of activity generally to the entire beta-lactam class of antibiotics including penicillins and cephalosporins [51]. The origin of SCC*mec* is thought to be originated from coagulase-negative staphylococcal species as there is no homologues of *mecA* present in methicillin-susceptible staphylococci. In recent years, a novel *mecA* homologue, *mecC* has been identified in both livestock and human in European countries. Similar to *mecA*, *mecC* codes for PBP2a with reduced affinity for methicillin and oxacillin, making them MRSA [52, 53].

2.3. Vancomycin resistance

As mentioned earlier, vancomycin is a gold standard antibiotic choice for MRSA infections. However, the emergence of vancomycin-intermediate *S. aureus* (VISA) (with a MIC value in the range of 3–8 μg/mL) and VRSA (with a MIC value ≥16 μg/mL) result in the failure of vancomycin treatment for MRSA infection. This antibiotic was first released in 1958. However, reduced vancomycin susceptibility in *S. aureus* was reported in 1997 in Japan [54]. VISA is also spreading to different parts of the world [38]. By comparison, the burden of VISA is relatively higher than VRSA, as the former is commonly associated with persistent infection, treatment failure and poor clinical outcomes. The molecular resistance of VISA is less-defined as compared to VRSA. Typically, VISA features increased cell wall thickness, reduced cross-linking rate, an increase of free D-alanyl-D-alanine residues in the peptidoglycan layers which provides more vancomycin binding, leading to an increased consumption of vancomycin while VISA remains unharmed [27, 55–57]. It is suggested that VISA involves accumulation of mutations, or rather, adaptation mechanisms in coping with the challenge of vancomycin. VRSA acquires complete resistance to vancomycin by obtaining plasmid(s) from vancomycin-resistant *Enterococcus* spp. that harbours *vanA* operon encoded on transposon Tn1546. VRSA maintains the resistance by retaining the original plasmid or by integrating Tn1546 from the enterococcal plasmid into staphylococcal resident plasmid. The *vanA* operon facilitates the synthesis of D-Ala-D-lactate instead of D-Ala-D-Ala peptidoglycan precursors. In doing so, vancomycin fails to bind hence leading to resistance observed in VRSA [57].

3. Bactericidal properties of natural products against drug-resistant *S. aureus*

Standard antibiotics treatment against drug-resistant *S. aureus* has failed in the clinical setting due to several causes as abovementioned. Interestingly, these resistant clinical isolates can be

killed by various naturally derived compounds and more promisingly, the antibiotic resistance exhibited by the bacteria can be reversed, and making them susceptible to the antibiotics again. In this section, we discuss the non-microbial natural products that showed bactericidal action against drug-resistant *S. aureus* and their potential to be used in combination with current antibiotics for its synergistic effects.

3.1. Potent natural products against drug-resistant *S. aureus*

Numerous natural products have shown potent antibacterial effects against *S. aureus*. Interestingly, these antibacterial actions are not limited to drug-sensitive wild-type *S. aureus*, but also extended to antibiotic-resistant *S. aureus*, including MRSA [58, 59], VISA [60], and VRSA [61]. Some of these natural compounds that showed promising bactericidal effects against drug-resistant *S. aureus* are summarised in **Table 1**. Due to the extensive repertoire of natural compounds against drug-resistant *S. aureus*, **Table 1** shows only those that are extracted from Pub-Med indexed publications, from year 2014 to 2017. These research articles reported the minimal inhibitory concentration (MIC) of the natural products against the drug-resistant *S. aureus* mainly MRSA using Clinical & Laboratory Standards Institute (CLSI) standard broth microdilution assay. As shown in **Table 1**, the MICs mostly range from micro to milligramme per millilitre. The bactericidal non-microbial natural products are derived from various sources including, plants, insects, animals, and fungi. These natural compounds have been reported to target and act on multiple bacterial targets such as cell wall [62, 63], pyruvate kinase [64], cell division [65], DNA topoisomerase [66], and efflux pump [67, 68]. These pharmacological targets are further discussed in Section 4.

3.2. Synergism of natural products and antibiotics

While serving as potent antibacterial agents alone, several studies have been carried out to investigate the potential of natural products to be used in combination with current antibiotics. This is particularly important against drug-resistant *S. aureus* which have shown resistance against several antibiotics. Natural compounds have been shown to reverse the antibiotic resistance. For instances, Akilandeswari and coworkers demonstrated that apigenin (AP) reversed the bacterial resistance of MRSA when used in combination with ampicillin and ceftriaxone [123]. The resulting MIC for ampicillin was shifted from 800 to 107 μg/mL, and the MIC for ceftriaxone was shifted from 58 to 2.6 μg/mL. Similarly, Mun and colleagues also showed that a plant-derived flavonol, morin reversed the oxacillin- and ampicillin-treated MRSA [124]. Essential oils derived from *Pituranthos chloranthus, Teucrium ramosissimum* and *Pistacia lentiscus* also reduced the resistance of MRSA to various antibiotics in Penicillins' group such as amoxicillin, piperacillin, and oxacillin [125].

Cumulative studies highlight the role of natural compounds in decreasing the reliance on antibiotics in bacterial treatment particularly in MRSA's management, hence preventing the emergence of antibiotic resistance. In addition, production of antibacterial agents from natural products might be more cost-effective than antibiotics production. With advent of modern biotechnology, mass production of these antibacterial products is feasible. More importantly, the manufacturing process allows genetic modifications (e.g. to improve biological activity,

Extract/ compound	Test strain	MIC	References
Curcumin	MRSA	217 μg/mL	[69]
Quinolone alkaloids	MRSA	8–128 μg/mL	[70]
Bee venom	MRSA	0.085–0.11 μg/mL	[71]
Magnolol and honokiol	MDR MRSA, MRSA	8–16 ppm	[72]
Quercus infectoria gall extracts	MRCoNS, MRSA	80–630 μg/mL	[73]
Kaempferia pandurata rhizome extracts	MRCoNS, MRSA	4–16 ppm	[74]
Mulinum spinosum extracts	MRSA	500–1000 μg/mL	[75]
Isothiocyanates from cruciferous plants	MRSA	2.9–110 μg/mL	[76]
Lichen	MRSA	3.9–500 μg/mL	[77]
Marinopyrrole A	MRSA	0.19–0.78 μM	[78]
6,6′-dihydroxythiobinupharidine	MRSA	1–4 μg/mL	[66]
Mature carpels of *Manglietiastrum sinicum*	MRSA	0.016–0.14 μM	[79]
Pentacyclic triterpenoids	MRSA	2–512 μg/mL	[80]
Psoralea corylifolia fruit constituents	MRSA	8–32 μg/mL	[81]
Thai longan honey	MRSA	12.5% (v/v)	[82]
Rubiaceae, Fabaceae, and Poaceae leaves extract	MRSA	5.5–388.4 μg/mL	[83]
Cinnamomum extracts	MRSA	19.5 μg/mL	[84]
Garcinia mangostana pericarp extracts	MRSA	17–20 μg/mL	[85]
MFM 501	MRSA	15.6–31.3 μg/mL	[86]
3′-demethoxy-6-*O*-demethylisoguaiacin	MRSA	12.5 μg/mL	[87]
Essential oils from *Schinus areira* leaves and fruits	MRSA	6–30 μg/mL	[88]
Spiromastixones A-O	MRSA	0.125–8 μg/mL	[89]
Hypericum riparium leaves extract	MRSA	6.54–18.5 μM	[90]
Demethyltexasin	MDR MRSA, MRSA	16–128 μg/mL	[91]
Oleoresin	MRSA	18.2–30 μg/mL	[92]
Thymoquinone	MRSA	8–16 μg/mL	[93]
Phenanthrene fraction	MRSA	8–64 μg/mL	[94]
Rhamnus californica and *Umbellularia californica* extracts	MRSA	3.3–6 mg/mL	[95]
Juncus and Luzula species	MRSA	9.75–156 μg/mL	[96]
E23 marine compound	MRSA	0.5–2 μg/mL	[97]
Piper betle extracts	MRSA	78–625 μg/mL	[98]
Letharia vulpine extracts	MRSA	31.25 μg/mL	[65]
Verrucosispora MS100047	MRSA	3.125–12.5 μg/mL	[99]
Micromonohalimanes B	MRSA	40 μg/mL	[100]
Pterospartum tridentatum extracts	MRSA	78.1 μg/mL	[101]

Extract/ compound	Test strain	MIC	References
N-3 substituted thiazolidine-2,4-dione derivatives	MRSA	6.25–12.5 μg/mL	[102]
Sapotaceae extracts	MRSA	45–97 μg/mL	[103]
Thymus daenensis	MRSA	25 mg/mL	[104]
Compositae extracts	MRSA	31.25 μg/mL	[105]
Roemerine	MRSA	32–64 μg/mL	[106]
Couroupita guianensis extracts	MRSA	62.5–156 μg/mL	[107]
Cotinus coggygria leaf extracts	MRSA	0.313–0.625 mg/mL	[108]
Eremophila alternifolia extracts	MRSA	10–20 μM	[109]
Baicuru	MRSA	39 μg/mL	[110]
Thymus bovei essential oil	MRSA	0.5 mg/mL	[111]
Formicamycins	MRSA	0.625–80 μM	[112]
Rumex aquaticus extracts	MRSA	192.3–463 μM	[113]
Endophenzine G	MRSA	2–128 μg/mL	[114]
Greek oregano isolates	MRSA	160–640 μg/mL	[115]
Macrocyclic bis(bibenzyl)s	MRSA	0.5–16 μg/mL	[116]
Acylquinic acids	MRSA	0.63–1.25 mg/mL	[117]
Houttuynia cordata poultice extracts	MRSA	0.11–1.76 mg/mL	[118]
Dandelion root extracts	MRSA	62.5–500 μg/mL	[119]
Solanioic acid	MRSA	1 μg/mL	[120]
Emodin	MRSA	32–64 μg/mL	[62]
Rhizoma coptidis	MRSA	1.2–2.84 mg/mL	[121]
Macrocyclic bis(bibenzyl)s	MRSA	0.5–32 μg/mL	[122]

MIC—minimal inhibitory concentration; MDR—multidrug-resistant; MRCoNS—methicillin-resistant coagulase negative *Staphylococcus aureus*; MRSA—methicillin-resistant *Staphylococcus aureus*.

Table 1. Antibacterial natural products against drug-resistant *Staphylococcus aureus*: An update from publication year 2014 to 2017.

Compound	Combination drug	Test strain	References
Curcumin	Oxacillin, ampicillin, ciprofloxacin, norfloxacin	MRSA	[127]
Curcumin	Gentamicin, amikacin, ciprofloxacin	MRSA	[128]
Lippia origanoides extracts	Neomycin, amikacin	MRSA	[129]
Grape pornace extracts	Oxacillin, ampicillin, nalidixic acid, ciprofloxacin, norfloxacin, levofloxacin, tetracycline, chloramphenicol	MRSA	[130]
Artocarpin	Ampicillin, norfloxacin, tetracycline	MRSA	[131]
Apigenin	Ampicillin, ceftriaxone	MRSA	[123]
Morin	Oxacillin	MRSA	[124]

Compound	Combination drug	Test strain	References
Bioactive fraction from *Duabanga grandiflora*	Ampicillin	MRSA	[132]
Bee venom	Ampicillin, penicillin, gentamicin, vancomycin	MRSA	[71]
Diosmetin	Erythromycin	MRSA	[64]
Brazilin	Amikacin, etimicin, gentamicin, streptomycin	MRSA	[133]
Sophora moorcroftiana genistein	Norfloxacin	MRSA	[134]
*Sophora moorcroftiana d*iosmetin	Norfloxacin, streptomycin, ciprofloxacin	MRSA	[134]
Medihoney	Rifampicin	MRSA	[135]
Magnolol and Honokiol	Oxacillin	MRSA	[63]
9EA-FC-B	Ampicillin	MRSA	[136]
Oxyresveratrol	Ciprofloxacin, gentamicin	MRSA	[137]
Zanthoxylum capense constituents	Ciprofloxacin	MRSA	[138]
Poncirus trifoliate extract	Oxacillin	MRSA	[139]
Juglans regia	Oxacillin	MRSA	[140]
Glabridin	Norfloxacin, oxacillin, vancomycin	MDR MRSA	[141]
Coumarins	Chloramphenicol, gentamicin, fosfomycin, levofloxacin, minocycline, piperacillin/ tazobactam, teicoplanin, vancomycin	MRSA	[142]
Polycarpol	Oxacillin, amoxicillin, vancomycin	MRSA, VISA	[143]
Linoleic and oleic acids	Erythromycin	MRSA	[144]
Salvianolate	Fosfomycin, erythromycin, piperacillin-tazobactam, clindamycin	MRSA	[145]
Phellinus baumii extracts	Oxacillin, cefazolin, cefepime, penicillin	MRSA	[146]
Epigallocatechin gallate	Oxacillin, tetracycline, ciprofloxacin	MRSA	[147]
Clerodane diterpene	Norfloxacin	MRSA	[67]
Carnosic acid	Gentamicin	MRSA	[148]
Ursolic acid	Ampicillin, tetracycline	MRSA	[149]
Anadenanthera colubrine extracts	Neomycin, amikacin	MRSA	[150]
Herbal extracts	Oxacillin, gentamicin	MRSA	[151]
Essential oils	Amoxicillin, tetracycline, piperacillin, ofloxacin, oxacillin	MRSA	[125]
Coumarin derivatives	Tetracycline, norfloxacin	MRSA	[68]

MDR—multidrug-resistant; MRSA—methicillin-resistant *Staphylococcus aureus*; VISA—vancomycin-intermediate *Staphylococcus aureus*; VRSA—vancomycin-resistant *Staphylococcus aureus*.

Table 2. Synergistic anti-*Staphylococcus aureus* effects of natural products and drugs.

solubility, stability, toxicity, production method, production cost and time, etc.) [41, 126]. Some of the challenges and limitation of using natural products as therapeutic modalities are discussed in Section 5.

4. Bacterial targets of *S. aureus* by natural products

In previous section, we discussed the anti-staphylococcal activities by various natural products alone and in combination with multiple types of antibiotics. As some of the mechanisms of antibiotic resistance have already been studied and reported, such as enzymes inactivation, antibiotics trapping, and efflux pumps, this information enables the anti-staphylococcal molecular targets of the natural products to be elucidated. These particular section summaries the molecular targets of natural products against drug resistant *S. aureus* such as bacterial cell wall and membrane, cell division protein FtsZ, pyruvate kinase, DNA topoisomerase, efflux pump proteins, and PBP2a. The reported pharmacological targets are depicted in **Figure 1**.

4.1. Cell wall and membrane

Cell wall of *S. aureus* is a popular pharmacological target of various antibiotics such as penicillins, cephalosporins, vancomycin, bacitracin, and others [152]. These antibiotics interfere with the cell wall biosynthesis and leading to death of the bacteria. Among all, peptidoglycan is the major cell wall components and has been targeted by various drugs [153]. Other cell wall components including adhesins, teichoic acids, immunodominant antigens, and cell wall enzymes are also being targeted by multiple antibiotics [154].

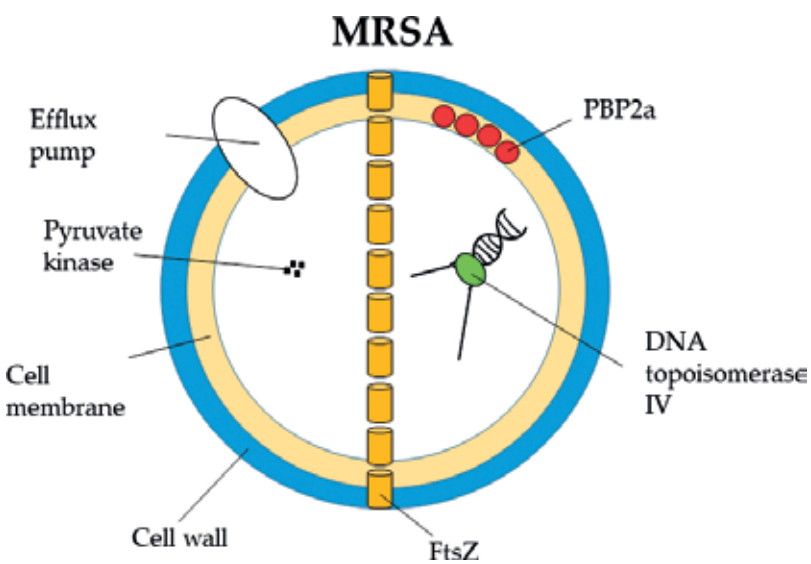

Figure 1. Pharmacological targets of drug-resistant *Staphylococcus aureus* from reported bactericidal natural products.

Similarly, a variety of bactericidal natural compounds also act on bacterial cell wall. For instances, Cao and coworkers showed that a natural compound emodin that targets MRSA could damage the cell wall and compromise the intracellular components. The cellular morphology was altered after the treatment when observed under transmission electron microscopy (TEM) [62]. Kim and colleagues also showed that magnolol targeted cell wall components to exert its pharmacological effect. In a mechanistic study, it has been shown that magnolol inhibited *mecI*'s pathway [63]. In addition, magnolol also targets various resistant genes, such as *mecA*, *femA*, and *femB* in mRNA form. It has also been shown that *Juglans regia* (English walnut) targeted the bacterial cell wall and resulted in the anti-staphylococcal effects [140]. While showing synergism in combination with antibiotics, apigenin was shown to compromise the cell membrane followed by subsequent leakage of intracellular constituents. This finding was demonstrated using TEM which showed significant morphological change of bacterial cell wall, shape, and plasma membrane [123].

4.2. Efflux pump

The function of efflux pumps of bacteria is to eliminate metabolites or materials that are potentially toxic and stress-inducing to the cells including antimicrobial compounds [155]. Hence, the bacterial efflux pumps have been known to contribute significantly to antimicrobial resistance by extruding a large number of antibiotics or drugs. They are often known as multidrug resistance (MDR) efflux pumps [155]. For decades, MDR efflux system has served as an excellent antibacterial target. Numerous promising candidates have previously demonstrated their potencies in targeting efflux pumps as the major mechanism to killing the bacteria [156, 157]. For examples, Wang and colleagues showed that genistein killed the MRSA by inhibiting NorA efflux protein when used in combination with drugs [134]. Mechanistic studies have also shown that various bactericidal natural compounds such as coumarin derivatives [68], linoleic and oleic acids [144], clerodane diterpene [67], and *Anadenanthera colubrina* (Cebil/Vilca) [150] acted on MRSA's efflux pump or proteins.

4.3. Penicillin-binding protein 2a (PBP2a)

PBP2a is encoded by *mecA* resistance gene and this gene can be acquired across different species for methicillin resistance [158]. Both PBP2a protein and *mecA* gene are emerging antimicrobial targets for therapeutics development [159, 160]. Various type of natural products targeting *mecA* gene or PBP2a have also been reported, these compounds include curcumin [161], tiliroside, pinoresinol, magnatriol B, and momorcharaside B [151], *Acalypha wilkesiana* (evergreen shrub) extract [136], and *Poncirus trifoliata* extract [139]. In combination with antibiotics, several natural compounds have also reduced the expression of PBP2a. For instances, Mun and colleagues showed that the combination of morin and oxacillin synergistically killed the MRSA depending on the PBP2a-mediated resistance mechanism [124]. Another study demonstrated that the combination of ampicillin and *Duabanga grandiflora* extract inhibited the PBP2a protein [132]. Hong and colleagues also showed that β-lactams and *Phellinus baumii* extracts synergistically killed the MRSA by targeting PBP2a [146].

4.4. Cell division protein FtsZ

FtsZ is a tubulin-like GTPase that recruit cell division proteins for new cell wall formation [162, 163]. Due to its pivotal role in cell division, it has been recognised as an important target for various antibacterial compounds or drugs including natural products. Liu and colleagues successfully developed several phenolic compounds targeting FtsZ of MRSA using a computer-aided simulation. These natural compounds showed potent bactericidal activities against MRSA [72]. It has also been shown that *Letharia vulpina* (lichen) extract possess antimicrobial activity by damaging cell membrane of MRSA, as well as disrupting cell division processes, possibly targeting FtsZ [65].

4.5. Other targets

Other bacterial proteins that are being targeted by natural products for antimicrobials discovery are pyruvate kinase (PK) and DNA topoisomerase IV. Pyruvate kinase serves as a catalyst to catalyse pyruvate and regulate carbohydrate metabolism [164] whereas DNA topoisomerase IV relaxes supercoiled DNA and performs decatenation events during DNA replication [165]. When used in combination with erythromycin, diosmetin drastically suppressed the MRSA PK activities in a dose-dependent manner. Chan and colleagues also speculated that the inhibition of PK could result in ATP deficiency and efflux pump malfunction [64]. Furthermore, Okamura and group demonstrated that a compound derived from *Nuphar japonicum* (water-lily) inhibited DNA topoisomerase IV of MRSA, but not DNA gyrase which is also carrying an important role in DNA replication [66].

5. Challenges and limitations

Despite great potentials shown by natural products of botanical origin, there is still a long way for them to be used for clinical application. Majority of these products function as supplements for their nutritional and immune-enhancing values, but none of these non-microbial derived natural products is FDA-approved, nor being used for treating bacterial infections. Several natural products antibacterials of microbial origins have been approved since 2010, including fidaxomicin, ceftaroline, dalbavancin, oritavancin, ceftolozane-tazobactam and ceftazidime-avibactam [166, 167]. Between 1980 and 2014, 59% of the total of 140 the FDA-approved antibacterials are originated from natural products or their derivatives, but none of them is originated from plants [166], despite the increasing evidences suggesting that plants may be promising antibacterials as discussed in this review. A few key challenges and limitations are highlighted and discussed in this section, including (a) design of antibacterial screening; (b) solubility and bioavailability of natural compounds; and (c) research directions towards clinical trials.

5.1. Design of antimicrobial screening

Antibacterial screening generally involves phenotypic screening relying on both Kirby-Bauer disc diffusion or broth micro-dilution methods. These methods are commonly used until

today due to the cost-effective and ease of preparation nature [168]. In disc diffusion method, antibacterial activity of an extract or compound is determined based on the presence of inhibitory zone on agar plates seeded with susceptible bacteria while broth micro-dilution method examines the MIC of the antibacterial that inhibits bacterial growth [169]. Very often, these methods are used in the initial antibacterial screening of crude extracts, which may comprise up to hundreds of compounds. This complexity may jeopardise the identification of true antimicrobial effects, leading to false negative results, as some active components may be of low abundance nature [170]. The exclusion of extracts and compounds that have high MIC values following initial screening means giving up on potential novel antibacterials. To overcome this, if crude extract is used, pre-fractionate followed by antibacterial screening to identify the most potent fraction is recommended. These fractions with promising results can be further sub-fractionated until potent compounds are identified. The fractionation technique usually involves the use of HPLC coupled to mass spectrophotometry [171]. By doing this, it reduces the chances of losing potent antibacterial during the screening step

In addition to the use of disc diffusion and broth-dilution methods, various techniques are currently used in the antibacterial studies. One such technique is the time-kill assay (also known as time-kill curve). In this technique, following the broth-dilution method, the bactericidal effects of different concentrations of the antibacterial agents (usually covering the ½ x MIC, 1 x MIC and 2 x of the MIC) at different time points, e.g. 0, 4, 6, 8, 10, 12 and 24 h, are assayed, revealing a time-dependent or a concentration-dependent antibacterial effects of these antibacterials [172]. At the moment, there is a lack of such studies in most of the reviewed articles. As the time-kill assay is able to provide a wealth of information on the dynamic interaction between antibiotics and the microbial strains, specifically *S. aureus* in this context, the inclusion of time-kill assay will further verify the antibacterial activity observed in natural products.

5.2. Solubility and bioavailability of natural products

One of the main limitations of adopting natural products for clinical applications is its solubility and bioavailability [161, 173]. This is highly related to the chemical properties, in particular aqueous solubility of the natural compounds. For examples, curcumin which is a polyphenolic compound, is known to have poor solubility in water, and the main solvents used are usually DMSO, DMF or ethanol [161]. The water insolubility has significant impact on its antibacterial effect and the reported biological action is further reduced under the physiological conditions [161]. There have been several studies demonstrating the reduced antimicrobial effects of natural compounds in the presence of normal human serum. Marinopyrrole A, which has previously shown potent antibacterial action against MRSA, showed approximately 256-fold higher MIC when tested in the presence of 20% serum [72]. The reduced activity could be due to the non-specific serum protein bindings and protein degradation due to metabolic enzymes and complements that largely affect the bioavailability. It has also been reported that curcumin showed reduced antibacterial activities against *S. aureus* when tested in the presence of human plasma and whole blood [174]. Similarly, human serum albumin has significantly decreased the bactericidal properties of curcumin [174, 175].

Numerous methods have been developed to overcome the solubility and bioavailability issues. Natural products loaded into nanocarriers such as nanoparticles, microemulsions,

micelles, *etc.* have improved the overall stability and bioavailability [176]. Incorporation of natural compounds such as resveratrol and thymol into liposomes has also increased the solubility and stability for their medicinal uses [173]. Furthermore, development of bioconjugates and nanoformulations also greatly improves the pharmacological action of natural products. This has been extensively reviewed for curcumin [161, 177].

5.3. Clinical trials

In the past decades, research organisations are de-prioritising natural products in their drug discovery programmes because of the costs associated with the development and licensure. For instance, between 1995 and 2001, Glaxo Smith Kline conducted 70 HTS campaigns, each worth approximately USD 1 million to identify only five potential antibacterial leads [178]. This early screening does not guarantee marketing and launching of these potential antibacterials as only approximately 30% of drugs, including natural products used as anti-infectives receive FDA approval [179]. Following initial *in vitro* testing, clinical (phase I to III) testing is required to ensure the efficacy and safety of new antibacterials on human subjects. The complexity of clinical trial adds another barrier to the development of new antibacterials [180, 181]. On one hand, pharmaceutical companies are faced with multiple regulatory bottlenecks such as increased stringency of trial design, increased demands regarding the design of phase III studies, and increased stringency of safety requirements for pre-licencing and post-licencing procedures of drugs [181]. On the other hand, bacteria are acquiring resistance at fast pace. It complicates clinical trials as these trials cannot be completed without a substantial number of the enrolled patients being infected with new, highly resistant strains. Clinical trials involving rare infectious diseases such as meningitis or endocarditis are most affected as these trials may take years and require multiple centres to complete [180]. Upon completion and success of clinical trials, pharmaceutical companies are required to file for approvals from the relevant agencies such as FDA in the US and European Medicines Agencies in the Europe. The entire process may take up to 15 years for the drug discovery to the launching stage [178]. The lack of interest and investment in antibacterial of natural sources reflects in the identification of only one such antibacterial agent, New Mexico Honey, as a decolonization agent for CA-MRSA abscess in the phase II clinical trial phase (ClinicalTrials.gov identifier number NCT00532324, accessed on the Dec 18, 2017).

In recognition of a lack of novel antibacterials in the clinical pipelines, FDA launched incentives such as Generating Antibiotics Incentives Now (GAIN) Act to foster the research and development of new antibacterial. For instance, granting five additional years of exclusivity to new antibacterials to the pharmaceutical companies, providing incentives for drugs used for treating serious and life-threatening infections, including *S. aureus*, and reducing new antibacterial drug application time to 6 months [182].

5.4. Future directions

The search for new antibacterial agent in natural products remains an exciting yet challenging task. Evidences show that regulatory agencies are working collaboratively with pharmaceutical companies in improving the development of new antibacterials from natural sources. The

combined efforts are the key in shaping the development and marketing of potent antibacterials in the coming years. Scientists working in the field, however, may play a bigger role in the discovery of novel antibacterials by addressing technical shortcomings of the screening of natural products for novel antibacterials.

One such aspect for consideration is to expand the antibacterial screening to include anti-virulence screening such as quorum sensing systems, biofilm formation and pilus adhesins. The investigation of anti-virulence rationalises that because anti-virulence drugs do not kill bacterial cells and thus exerting less selective pressure for resistance. It is believed that the development of resistance is slower compared to bactericidal agents. Anti-virulence would constitute a valuable alternative to bactericidal agents [183, 184]. Anti-virulence of natural product such as anti-quorum sensing of goldenseal (*Hydrastis canadensis* L.) [185], anti-biofilm of dihydrocelastrol and dihydrocelastryl acetate present in many plants [186] in MRSA have been reported. This area of research is still lacking, in-depth investigation on anti-virulence potentials and solid evidence of slow resistance rate is still required.

Another challenging aspect of natural product not mentioned earlier is low bioavailability of natural products, creating inconsistent results between preclinical and clinical studies [187–189]. To overcome this challenge, scientists are exploring the incorporation of nanoparticles into a delivery system for natural products in order to increase therapeutic effects of natural products [190]. Preclinical successes of curcumin-nanoparticles in inhibiting *in vitro* growth of *S. aureus* [191] and MRSA and enhancing wound healing in in vivo murine wound model [192] have been documented thus far. This emerging field holds promises for natural products in treating bacterial infections. However, drug targeting using nanoparticles remains a challenge, toxicity and safety needs further in-depth evaluations.

6. Conclusions

Non-microbial natural products have shown promising bactericidal activities against drug-resistant *S. aureus*. The mechanisms of bacterial killings are under investigation and great efforts are being made to evaluate their antibacterial activities in clinical trials. This chapter provides an important update on the anti-staphylococcal activity of natural products against *S. aureus* and the underlying challenges are highlighted. These issues need to be addressed in order to transform the antibacterial natural products into clinically useful antibiotics in the future.

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Chapter 2

Typification Methods and Molecular Epidemiology of *Staphylococcus aureus* with Methicillin Resistance

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Abstract

Recent interest in the study of *Staphylococcus aureus* derives from the high frequency of antibiotic-resistant strains that cause frequent outbreaks of infection, especially methicillin-resistant *S. aureus* (MRSA). The objective of this chapter was to study the population genetic structure and the origin of MRSA isolation. Classification of staphylococcal cassette chromosome mec (SCCmec) is the most important method to identify and define the *S. aureus* methicillin-resistant clonal nature. Molecular epidemiological studies have demonstrated dissemination patterns of few strains which are responsible for the important worldwide problem. There is a predominance of pandemic clones of MRSA associated to hospital-acquired infections (HA-MRSA) which has been replaced today by community-acquired strains (CA-MRSA). Understanding the epidemiology and clonality of *S. aureus* infections has important implications for future efforts to control of the emergence of multidrug-resistant strains and the spread of clones resistant and sensible to methicillin.

Keywords: methicillin-resistant *S. aureus*, clonal complex, molecular epidemiology, classification, typing

1. Introduction

Staphylococcus aureus is one of the most common pathogenic organisms responsible for a wide variety of infectious syndromes [1, 2]. Significant increase in the prevalence and emergence of methicillin-resistant *S. aureus* (MRSA) is a serious public health concern and has a dramatic negative impact on medical practices [3, 4]. Therefore, identification of MRSA strains is important for both clinical and epidemiological implications.

On the other hand, it is important to carry out the typing of *S. aureus* to defining the occurrence of an epidemic, monitoring the transmission of the organism among carriers, contributes to the evaluation of nosocomial infection outbreaks, recurrent infection and the use of the appropriate measures in a local environment. In addition, determining the origins of these strains can help in delineating their circulation among different environments [5, 6].

Different genotypic and phenotypic methods have been developed for this purpose. However, each method has its own advantages and disadvantages, so the optimal method of bacterial strain typing depends on the objectives of data collection and available resources [7–9]. Thus, techniques with high discriminatory power with the ability to distinguish between epidemiologically unrelated bacterial strains are adequate for carrying out locally restricted epidemiological studies or epidemic outbreaks. While, the sequence-based techniques that analyze more stable genetic markers are more appropriate for recognizing ancestral relationships between the bacterial strains [9].

In this chapter, we expose the methods of detection and typing of *S. aureus* and MRSA isolations, through which progress has been made in understanding the molecular epidemiology of the bacterium.

2. Identification of *S. aureus*

The high pathogenicity of *S. aureus* causes frequent nosocomial and community infections, so its isolation and rapid identification is extremely important for timely treatment [1, 2]. The diagnosis of diseases caused by *S. aureus* should be based first of all on the clinical picture and then confirm with a culture where it is isolated [6, 10].

Gram staining of the colony and tests for the production of catalase and coagulase are the ideal techniques that allow the rapid identification of coagulase-positive *S. aureus* [11, 12]. Another very useful test for its identification is the production of thermostable deoxyribonuclease [12].

2.1. Latex agglutination test

S. aureus produces two forms of coagulase: bound coagulase, or "clumping factor", can be detected by carrying out a slide coagulase test, and free coagulase can be detected using a tube coagulase test. Hemagglutination test with fibrinogen-sensitized sheep erythrocytes is used for the detection of clumping factor.

Also slide agglutination test with plasma-coated latex is used for the simultaneous detection of clumping factor and protein A. In principle, plasma contains fibrinogen, which has the capacity to bind to clumping factor, and immunoglobulin, which has the capacity to bind to protein A through its Fc fragment. Hence, the presence of either clumping factor or protein A on the bacterial cell results in co-agglutination of cells and latex particles [13].

There are variants of the agglutination tests that use different surface antigens, specific for *S. aureus*, which contributes to an increase in the sensitivity of the tests, especially for some *S. aureus* isolates that produce relatively small amounts of coagulase or protein A [14].

On the other hand, the techniques based on the molecular identification of *S. aureus* like fluorescent in situ hybridization (FISH) use artificial probes labeled with fluorescent molecules and specific for *S. aureus* are applied in order to differentiate this species [15]. Molecular tests based on the PCR method, which demonstrate the genes which code nuclease (*nuc*), coagulase (*coa*), protein A (*spa*), *fem*A, *fem*B, sa442, 16SrRNA and fibrinogen-binding proteins [16–18].

3. Detection of MRSA isolates

The fact that methicillin resistance is undoubtedly related to the importance of the *mec*A gene, makes it possible to create molecular tests relatively quickly for definite proof of MRSA.

S. aureus acquires methicillin resistance through *mec*A gene that is responsible for the synthesis of a 78-kDa protein, called penicillin-binding protein 2a (PBP2a). PBP2a substitutes other PBPs, that catalyze the transpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains, but its active site blocks binding of all β-lactams but allows the transpeptidation [19, 20].

The *mec*A gene is regulated by the repressor MecI and the trans-membrane β-lactam-sensing signal transducer MecRI, both of which are transcribed divergently. However, in the absence of a β-lactam antibiotic, MecI represses the transcription of both *mec*A and *mec*RI-*mec*I. In the presence of a β-lactam antibiotic, MecRI is cleaved autocatalytically, and a metallo-protease domain, which is located in the cytoplasmic part of MecRI, becomes active. The metallo-protease cleaves MecI bound to the operator region of *mec*A, which allows transcription of *mec*A and subsequent production of PBP2a [19].

The *mec*A gene is part of a genomic island designated staphylococcal cassette chromosome mec (*SCCmec*) [21].

SCCmec elements integrate sequence at the bacterial chromosomal attachment site (*attBscc*) that is located near the origin of replication, at the 3′ end of an open reading frame of unknown function, termed *orfX*, well conserved among both MRSA and MSSA strains [21–23].

The attachment site contains a core 15-bp sequence, called the integration site sequence (ISS) that is necessary for ccr-mediated recombination [21]. *SCCmec* integrated into the chromosome consists of *mec* complex, composed of *mec*A operon, *ccr* gene complex, composed of cassette chromosome recombinase (*ccr*) gene(s) and three regions bordering the ccr and mec complexes, designated as joining (J) regions, that is, with composition presented as follow: (*orf*X)J3-*mec*-J2-*ccr*-J1 [21, 22].

The 2.1-kb *mec*A gene is regulated by the repressor MecI and MecRI that are transcribed divergently. In the absence of a β-lactam antibiotic, MecI represses the transcription of both *mec*A and *mec*RI-*mec*I. In the presence of a β-lactam antibiotic, MecRI is activated by autolytic cleavage and cleaves MecI bound to the operator region of *mec*A, which allows transcription of *mec*A and subsequent production of PBP2a [21–23]. Both *mec*I and *mec*RI can be truncated by insertion sequences IS431 or IS1272, and these results in derepression of the *mec*A gene [24].

There is a *mec*A homolog, termed *mec*C, which is only ~69% identical to conventional *mec*A at the DNA level, and the encoded PBP2a/2′ is ~63% identical at the amino acid level. Similar

to conventional *mec*A, *mec*C is located within a *SCCmec* element inserted into the 3′ region of orfX but it had divergent ccrA and ccrB recombinases (belonging to the ccrA1 and ccrB3 groups and representing a novel combination of recombinase groups designated type 8 ccr), divergent *mec*A regulatory genes (mecI/mecR) and the absence of one of the three joining regions (J3) that are normally present [25].

In the identification of MRSA, MRSA Screen Latex agglutination test is a slide agglutination assay to detect penicillin-binding protein (PBP2a) from isolates of Staphylococci. The tool contains latex particles sensitized with a monoclonal antibody against PBP2a [26]. The Cefoxitin Disc Diffusion and Oxacillin Agar Screen developed on Muller Hinton agar plates are the phenotypic tests used routinely [27–29]. Methods based on detection of the *mec*A gene, the PCR method are also used in many laboratories [30, 31].

4. Typing of *S. aureus*

Everyday, the techniques of bacterial molecular typing become more available. Optimal typeability, a high degree of reproducibility, adequate stability and unprecedented resolving power characterize the "gold standard" typing technique [8].

4.1. Phenotypic methods

The conventional methods used for the typing of *S. aureus* and especially of the MRSA strains emerged in the 1950s and 1960s, all being phenotypic methods, among these methods, biotype, serotyping, fagotipage and resistograms (resistance to chemicals and dyes) were highlighted [8, 9]. In the case of resistograms, ethidium bromide, cadmium nitrate, phenyl mercuric acetate and mercuric chloride have been used on the basis of the susceptibility pattern produced.

4.2. Serotyping

Serotyping is based on fact that strains of same species can differ in the antigenic determinants expressed on the cell surface such as lipopolysaccharides, membrane proteins, capsular polysaccharides, flagella and fimbriae exhibit antigenic variations. Strains differentiated by antigenic differences are known as 'serotypes'.

Serotyping of capsular polysaccharides in *S. aureus* has allowed to establish a total of 11 capsular types, but 85–90% of clinical isolates belong to just two of them. For example, in SARM only serotype 5 or 8 is detected.

This method has limited application in epidemiological studies because a large number of unrelated isolates belong to a small number of capsular serotypes [32].

4.3. Phage typing

Strains can be characterized by their pattern of resistance or susceptibility to a standard set of bacteriophages. This relies on the presence or absence of particular receptors on the bacterial

surface that are used by the virus to bind to the bacterial wall. This method is used to type isolates of *S. aureus* and is referred as 'phage types' and was standardized by the International Subcommittee on Phage Typing of Staphylococci [33].

Human strains of *S. aureus* are classified according to their susceptibility to a set of set of 23 phages (group I—29, 52, 52A, 79 and 80; group II—3A, 3C, 55 and 71; group III—6, 42E, 47, 53, 54, 75, 77, 83A, 84 and 85; group V—94 and 96; not classified—81 and 95) internationally accepted for typing. The technique requires maintenance of biologically active phages and is available only at reference centers. This technique has been reported to be valuable in the identification of known epidemic strains among endemic strains and is preferred as first line approach in epidemiological investigation of MRSA strains [32]. Phagotyping also has limited application since a significant number of isolates are not susceptible to bacteriophages and it is not possible to apply this method to them [33].

4.4. Biotyping

Biotyping is a rapid and inexpensive method that makes use of the pattern of metabolic activities expressed by an isolate, colonial morphology and environmental tolerances and strains are referred to as "biotypes".

Devriese proposed a simplified biotyping system for the typing of *S. aureus* strains on the basis of the evaluation of synthesis of fibrinolysin and β-hemolysin, coagulation of bovine plasma and type of growth on medium containing crystal violet [34]. This method allows to differentiate *S. aureus* isolates from host specific (HS) ecovars: human, bovine, ovine and poultry biotypes; the strains which could not be classified into any of these biotypes on the basis of their properties were referred to as non-host-specific (NHS).

In the 1990s, Isigidi et al. described a new biotype, P-like pA+ (poultry-like protein A positive), and was tentatively designated as an "abattoir" biotype [35]. The introduction of an additional biochemical test, protein A production permitted showed typical properties of the poultry biotype but differed from it in terms of the synthesis of protein A. This biotype was initially described solely in meat products and meat industry workers. In 2016, Piechowicz and Garbacs, revealed that the P-like pA+ biotype strains can be also present in hospitalized patients and extra-hospital carriers with greater genetic variability [36].

This method has been useful in tracing the origin of *S. aureus* isolates in food animal and food industry and the probable source of contamination of foods by *S. aureus*. Kitai et al. showed that retail raw chicken meat in Japan is frequently contaminated with *S. aureus* strains belonging to the human and poultry biotypes [37].

Hakimi et al. showed that different animal ecovars were characterized among human and bovine raw milk isolates, confirm the possibility of the transmission of *S. aureus* strains among humans and different animal species, and this can be very important, especially when such strains carry antibiotic resistance genes [38].

Hennekinne et al. investigated the genotypic discrimination between *S. aureus* strains assigned to different biotypes with PFGE patterns showing a strong correlation between

pulsotypes and biotypes, and confirm the abattoir biotype as an individual group [39]. However, strain discrimination is limited, variation in gene expression, due mainly to point mutations is the most common reason for isolates that represent single strain to differ in one or more biochemical reactions.

4.5. Antimicrobial susceptibility typing (antibiogram)

Phenotyping methods also include examination of susceptibility to antimicrobes, which has the practical value in recommending treatment for the infection and as a strategy in the control of resistance to antibiotics [28, 29].

A common method for the detection of MRSA employs the technique of diffusion in hypersaline Mueller Hinton agar, with a disc of 1 μg of oxacillin, incubating at 35°C for 24–48 h (halo inhibition ≤10 mm) [40] or the study of the minimum inhibitory concentration (MIC) by means of an E-test with oxacillin strip. Additionally, it has been demonstrated that cefoxitin (cefamycin) in vitro, induces the production of PBP2a in strains of sensitive methicillin *S. aureus* [27]; therefore, the disc diffusion method using cefoxitin (FOX 30 μg) has proven to be a good assay for the detection of low level resistance to oxacillin in strains of *S. aureus*. Currently, the cefoxitin disc is used as a substitute for oxacillin for the phenotypic detection of MRSA strains [29].

Antibiogram typing profiles or antibiotypes involves comparison of susceptibilities of isolates to a range of antibiotics. Isolates differing in their susceptibilities are considered as different strains. An unusual pattern of antibiotic resistance among isolates from multiple patients is considered as an indication of an outbreak [41].

Antibiotic susceptibility patterns has been the main typing tool in many hospital outbreaks since the technique is widely available and standardized. With the use of the antibiogram, it has been shown that the pattern of susceptibility to antibiotics varies according with time and geographical location [42]. However, antibiotic resistance patterns are also, to some extent influenced by the local environment, selective antibiotic pressure, acquisition and loss of plasmids carrying resistance genes and various other genetic mechanisms.

One way to optimize the antibiotype to evaluate the clonal relationship between two bacteria is given by the quantitative antibiogram. This mathematical technique proposed for Giacca et al. is based on disc zone sizes, in order to assess the probability of two or more clinical isolates to be the same strain [42]. Method uses the comparison of the diameters of the inhibition rings in the disc diffusion technique (Kirby Bauer) [41]. Antimicrobials are selected with greater variation for the strain under study, to allow better discrimination. The result of the summation of the inhibition zones of a bacterial isolation is evaluated and compared with the other isolation by using a coefficient of similarity.

Similarity of strains is reported in a dendrogram, in which strains are successively fused. Strains that share a common susceptibility pattern are considered a "cluster" [42].

Although useful as a screening method for detecting certain resistance profiles and for selecting potentially useful therapeutic agents, conventional antimicrobial susceptibility testing methods are insensitive tools for tracing the spread of individual strains within a hospital or region [8, 9].

4.6. Molecular typing techniques of *S. aureus*

In order to examine more thoroughly the molecular evolution of *S. aureus*, especially of MRSA and its spread in world terms, several molecular typing techniques have been developed [5, 6, 8, 9]. These methods involve the study of the microbial DNA, the chromosome and plasmid, their composition, homology and presence or absence of specific genes. These techniques are more frequently applied and better appreciated than the phenotypically oriented approaches in taxonomy, epidemiology and evolutionary studies that have enhanced our understanding of disease epidemiology and provided insight into the evolution of bacterial pathogens [5].

4.7. Plasmid profile analysis

Plasmid analysis was the first molecular technique used for epidemiological investigation of MRSA and MSSA [43].

In this technique, the isolates are differentiated according to the number and sizes of plasmids carried by an isolate, but its reproducibility suffers due to the existence of plasmids in different molecular forms such as supercoiled, nicked or linear, each of which migrates differently on electrophoresis.

The plasmids contain resistant genes against a number of antimicrobial agents, so it has been useful to assess the relatedness of individual clinical isolates of *S. aureus*, in the epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance [44].

Agbagwa and Jirigwa determined the antibiotic-resistant pattern and plasmid profile of *S. aureus* obtained from wound swabs and found similar antibiotic resistance pattern, while different plasmid sizes was observed in the isolates [45]. Jaran also found no direct correlation between the patterns of antibiotic resistance and plasmid profiles in clinical isolates of *S. aureus* in hospitals of Saudi Arabia [46]. This disparity can be due to R-plasmids of different sizes which are also responsible for the presence of multiple resistances.

The technique has not been found to be very useful for the investigation of outbreak infections because the plasmids can be spontaneously lost or readily acquired, related strains can exhibit different plasmid profiles. Also, certain genes are contained in transposons that can be readily acquired or deleted. Some isolates may lack plasmids and will not be typeable by this method [44, 45].

4.8. Chromosomal DNA analysis

4.8.1. Ribotyping

Methods designed to recognize restriction fragment length polymorphisms (RFLP) using a variety of gene probes, including rRNA genes (ribotyping) and insertion sequences. The probes generally used are either labeled with radioisotopes or are biotinylated. In this technique, the choice of restriction enzyme used to cleave the genomic DNA, as well as the probes, is crucial. Restriction enzyme *Eco*R1 has been found to be comparatively more useful than other enzymes in producing a good number of bands [47].

The southern blot hybridization of MRSA fragments after RFLP may contain genes specific for staphylococcus in the form of a probe, including the *mec*, transposon Tn554, *agr*, *aph*(2″)-*aac*(6′) (gene resistance to aminoglycoside).

4.8.2. Pulsed-field gel electrophoresis (PFGE)

PFGE is a technique based on digestion of purified chromosomal DNA with restriction enzyme *Sma*I, generating large fragments of DNA that are separated in agarose molds and detection of fragments by PFGE. Migration of large DNA fragments (10–800 kbp) through the electrophoresis gel is realized by use of an electrical field which changes direction over graded time intervals, so minimizing the overlapping of fragments [47, 48]. The obtained PFGE patterns are evaluated with the Dice coefficient and unweighted pair-group matching analysis (UPGMA) settings, according to the criteria described by Tenover et al. [49]. For the application of these criteria, it will be required that the digestion with the enzyme generates a minimum of 10 bands.

In the USA, a national PFGE-based typing system for *S. aureus*, designated as pulsed-field types USA100 through USA1200 that has been an important tool to facilitate the exchange of PFGE strain typing data and epidemiologic information among reference laboratories has been established [50].

4.8.3. Polymerase chain reaction (PCR)-based typing methods

To facilitate the process of the analysis of *S. aureus* isolates, polymerase chain reaction (PCR)-based typing methods have been developed for their simplicity and the obtaining of fast results. With this technique, it is possible to generate DNA profiles that can be analyzed by gel electrophoresis or DNA sequence analysis [51].

4.8.4. PCR-restriction fragment length polymorphisms (PCR-RFLP)

This typing technique involves the amplification of a defined fragment of DNA and subsequent digestion of the amplified product with a restriction enzyme. Variations in the number and sizes of the fragments detected are referred to as restriction fragment length polymorphism (PCR-RFLP). These fragments are separated on agarose gel electrophoresis and strains can be characterized by their restriction profiles [5].

PCR-RFLP of genes coding for two species-specific proteins, coagulase (*coa*) and staphylococcal protein A (*spa*), have been used to discriminate MRSA strains [8, 52].

4.9. DNA sequence analysis-based typing methods

DNA sequence analysis is an objective genotyping method as the genetic code is highly portable, easily stored and can be analyzed in a relational database [5, 8].

4.9.1. Multilocus sequence typing (MLST)

MLST is a well-established method to study bacterial populations exhibiting sufficient nucleotide diversity in a small number of genomic loci [53].

Due to the specific characteristics of *S. aureus*, it is very suitable to follow clonal evolution of MRSA and MSSA, monitoring genetic changes over long periods of time and in different geographical areas, which has allowed to have a global epidemiological view of the bacterium [54].

The method is based on nucleotide sequences analysis of 0.5-kb fragments from seven housekeeping genes of *S. aureus*: *arcc*, *aroe*, *glpF*, *gmk*, *pta*, *tpi* and yqil. They code the following enzymes, respectively: carbamate kinase, shikimate dehydrogenase, glycerol kinase, guanylate kinase, phosphate acetyltransferase, triosephosphate isomerase and acetyl-coenzyme A acetyltransferase [55]. Since mutations accumulate slowly in housekeeping genes, the MLST scheme is used to delineate clusters of closely related strains.

The sequencing of each gene allows obtaining the allelic profile or sequence type (ST) profile, which are given by the alleles of the seven genes.

The Iberian clone is the most frequent with a MLST profile 3-3-1-12-4-4-16, and belongs to ST247 (www.mlst.net).

The analysis of the MRSA structure is based on the determination of the ST and the *SCCmec* type and is grouped into clonal complexes (cc). Isolates of *S. aureus* are assigned to the same clonal complex when 5 of 7 genes have identical sequences. This analysis is carried out using the 'eBURST', a computer program (based on repeated sequences), developed at the University of Bath in the UK that detail how MRSA spread [56]. Databases containing MLST and associated data from hundreds or thousands of isolates can be accessed via the internet (http://www.mlst.net/ and http://pubmlst.org/) [57].

MLST has provided numerous insights into the epidemiology and population genetics of bacteria and is an excellent tool for investigating the clonal evolution of MRSA. However, MLST is not suitable to characterize the differences in strains within an outbreak as its power to resolve small evolutionary differences is too low. In addition, the costs of sequencing currently limit their routine uses for most epidemiological studies [53–55].

4.9.2. Single-locus sequence typing

Single-locus sequence typing (SLST) is used to compare sequence variation of a single target gene. The genes selected are usually of short sequence repeat (SSR) regions that are sufficiently polymorphic to provide useful resolution. The technique is simple, rapid and highly reproducible [5, 8].

4.9.3. Typing coagulase (coa)

The coagulase gene amplification discriminatory power relies on the heterogeneity of the region containing the 81 bp tandem repeats at the 3′coding region of the coagulase gene which differs both in the number of tandem repeats and the location of *Alu*I and *Hae*III restriction sites among different isolates [52]. Variations in the sequence of genes coding for coagulase (*coa*) showed a good correlation with PFGE typing.

4.9.4. Spa typing

The *spa* gene contains three distinct regions: Fc, X and C [52]. *Spa* typing is a single-locus typing based on sequencing of short sequence repeat (*ssr*) regions of the polymorphic X region of the protein A gene (*spa*) of *S. aureus* [60]. The polymorphic X region consists of up to 12 units each with a length of 24-bp variable-number tandem repeat (VNTR) within the 3′ coding region. The composition of the repeating fragments is presented in letters, in that a group of fragments in a certain isolate comprises the "*spa* repeat" code. The repeating fragments are also marked by a number, in view of their large number and for easier data processing.

Isolates are assigned to particular *spa* types using the *spa* typing website (http://www.spaserver.ridom.de). Several studies have demonstrated that *spa* typing is highly discriminatory, and useful in both local and global epidemiological studies [58].

In addition to its use as a marker, the number of repeats in the region X of *spa* has been related to the dissemination potential of MRSA, with higher numbers of repeats associated with higher epidemic capability; it detects genetic microvariations and may be used in phylogenetic studies, where genetic macrovariations are key [58].

4.9.5. SCCmec typing

The first *SCCmec* element was identified in Japanese *S. aureus* strain and shortly after two additional *SCCmec* were determined; these three *SCCmec* elements were classified as types I–III [23, 61]. Subsequently, two other *SCCmec* were described: *SCCmec*IV [59] and *SCCmec*V [60].

Currently, 11 *SCCmec* types are known: *SCCmec*VI, *SCCmec*VII, *SCCmec*VIII, *SCCmec*IX, *SCCmec*X, *SCCmec*XI [61–63].

Variation in these *SCCmec* types has made the basis for differentiation among MRSA strains, and each *SCCmec* type encodes for resistance to different antibiotics. *SCCmec* types I (34.3 kb), IV (20.9–24.3 kb) and V (28 kb) encode exclusively for resistance to β-lactam antibiotics [63]. *SCCmec* types II (53.0 kb) and III (66.9 kb) determine multiresistance, as these cassettes contain drug resistance genes on integrated plasmids: pUB110, pI258, pT18 and a transposon Tn554 that confers additional resistance to kanamycin, tobramycin, bleomycin, heavy metals, tetracycline, lincosamide and streptogramin [23, 62].

The *mec* complex also contains the insertion element IS431*mec*, which has been frequently associated with genes encoding resistance to various antibiotics and mercury; in some isolations is also the IS1272 [24]. When regulatory genes *mec*RI (on *SCCmec* types I, IV and V) or *mec*RI and *mec*I (on *SCCmec* types II and III) are intact and fully functional, they appear to confer greater repression on the expression of PBP2a [21, 22, 64, 65].

It has been reported that the *SCCmec* is not restricted to the mobility of the *mec*A gene; he has additional elements, called non-mec, that contribute to the survival and pathogenic potential of *S. aureus*. Among the non-mechanical elements are sequences coding for resistance to heavy metals such as mercury (*SCCmer*) or fusidic acid (SCC MSSA 476, Staphylococcal cassette chromosome methicillin-susceptible *S. aureus*) sequences for biosynthesis capsular (SCCcap1), for the protection of DNA by modification-restriction systems (SCC CI) and for the catabolism of arginine (ACME, arginine catabolic mobile element) [23, 24].

J regions from different *SCCmec* elements are unique to particular types of ccr-mec gene complex combinations and variations of these regions within the same ccr-mec gene complex combination are specific for *SCCmec* subtypes [66, 67]. In the case of *SCCmec*VII and *SCCmec*IX, ccr gene complex positioned between J3 and J2 regions and the *mec* gene complex between J2 and J1 regions is presented [61].

In addition to the *SCCmec* types, several variants of *SCCmec* have been described. Depending on the structural diversity of *mec*I-mecR1 region, six major classes, A–E, of *mec* complexes have been distinguished [67]: Class A, which contains intact *mec* gene complex; Class B, where mecR1 is truncated by insertion sequence IS1272; Class C1, where *mec*R1 is truncated by insertion sequence IS431 having the same direction as the IS431 downstream of *mec*A; Class C2, where mecR1 is truncated by insertion sequence IS431 having the reverse direction to the IS431 downstream of *mec*A; Class D, where *mec*R1 is partly deleted but there is no IS element downstream of Δ*mec*R1 and has been observed in *S. caprae* only. The sixth complex obtained of genome sequence of the bovine *S. aureus* isolate LGA251 assigned as class E [68].

In relation to the genes of the *ccr* complex are designated *ccr*A1 and *ccr*B1 (in *SCCmec* type I), ccrA2 and ccrB2 (in *SCCmec* types II and IV), ccrA3 and ccrB3 (in *SCCmec* type III), ccrA4 and ccrB4 (in *SCCmec type* IV of MRSA strain HDE288) and ccrC (in *SCCmec* type V) [61, 66].

The method of Oliveira and de Lencastre is the most used and cited, which uses the multiplex PCR method for *SCCmec* types I–IV, to detect six gene loci and the *mec*A gene in the *SCCmec* complex [91]. Zhang et al. used a multiplex PCR for the characterization of *SCCmec* types I–V and differentiate between subtypes of *SCCmec* IV (a–d) [69].

Classification scheme of Chongtrakool et al. for the nomenclature of *SCCmec* is based on the *ccr* genes (indicated by a number) and the mec complex (indicated by an upper-case letter). Application of this nomenclature results in *SCCmec* type 1A (type I), type 2A (type II), type 3A (type III), type 2B (type IV) and type 5C (type V). Differences in the J1 region and the J2–J3 regions are then designated with numbers, for example, *SCCmec* type 2B.2.1 (type IVb). The *ccr* genes and the J regions are numbered in chronological order according to their discovery [70].

A combination of two approaches like *SCCmec* typing along with MLST is recommended for reliable typing for multicentre surveillance, inter-hospital and international transmission and evolution of MRSA strains [71].

Studies have found that healthcare associated MRSA (HA-MRSA) strains contain mainly *SCCmec* type I, type II and type III, while community-associated MRSA (CA-MRSA) strains contain type IV and type V cassettes, although several variants have also been reported [72].

4.9.6. Toxin gene profile typing

Studies have shown that MRSA strains possess more toxin genes as compared to MSSA strains. The pathogenicity of *S. aureus* is determined by a variety of bacterial cell wall surface components and exoproteins including toxic shock syndrome toxin (TSST-1), enterotoxins, exfoliative toxins and Panton Valentine leukocidin (PVL) [73].

The *PVL* genes are predominantly associated with *S. aureus* strains that cause community-acquired infections, including skin and soft-tissue abscesses, necrotizing pneumonia and

invasive osteomielitis [74]. These genes are carried on bacteriophages and are easily transferred between lineages. Thus toxin gene profile of the strains can be used as an important epidemiological marker for typing of MRSA strains [75]. MRSA strains isolated from different geographical areas have shown to possess distinct toxin gene profiles. Studies on toxin gene profile of MRSA have reported that most of the CA-MRSA possess genes for PVL toxins and may have evolved from the established CA-MSSA (community-acquired methicillin sensitive *S. aureus*) strains [76]. Of the various methods available, multiplex PCR technique is recommended for detection of toxins in MRSA. It is rapid, reproducible relatively inexpensive, easier to interpret and provides a high degree of discrimination. The technique is useful for studying the chromosomal diversity and evolutionary history of MRSA strains [75].

Today, a greater discrimination such as provided with whole-genome sequencing (WGS) and single-nucleotide polymorphism (SNP) analysis would be useful. High-resolution phylogenetic and phylogeographic (phylodynamic) analyses based on genome-wide SNP data are a powerful tool to infer the origin and test spatiotemporal hypotheses of MRSA spread [77, 78].

The evolutionary rate of MRSA genome-wide SNPs estimated by Gray et al. demonstrates that bacterial genomes can indeed contain sufficient evolutionary information to elucidate the temporal and spatial dynamics of transmission. In the case of HA-MRSA ST239 strain, phylogeographic analyses statistically supported the role of human movement in the global dissemination of this strain [79].

5. Genetic structure of the population of *S. aureus*

The molecular typing techniques have been used in combination to elucidate and study the population structure of *S. aureus* [80–82].

Accordingly, combinations of DNA band-based techniques with DNA sequence-based techniques are frequently used to differentiate between MRSA strains at the local and the international levels [8, 55, 56].

These techniques confirmed the notion that *S. aureus* is a polymorphic species with a clonal population structure [55, 56, 82] that does not undergo extensive recombination, diversifies largely by nucleotide mutations and shows a high degree of linkage disequilibrium (nonrandom associations between genetic loci).

Molecular evolution of MRSA has been favored by horizontal gene transfer [56] and clonal dissemination of certain strains [83–85].

Although *S. aureus* is considered to be an opportunistic pathogen, it is possible that certain clones are more prone to cause invasive disease than are others, due to the presence of virulence factors that increase their chance of gaining access to normally sterile sites [86, 87].

MLST group strains into sequence types (STs) has been used in conjunction with PCR analysis of *SCCmec* element to define the clonal type of MRSA strains (CCs) [55, 58]. Enright et al. [84] using both methods found five clonal complexes found among the population from Southern

Europe, the USA and South America, and defined as groups of isolates from more than one country with the same ST and *SCCmec* type, belonged to one of five clonal complexes, namely the Iberian (ST 247-MRSA-IA), Brazilian (ST239-MRSA-IIIA), Hungarian (ST239-MRSA-III), New York/Japan (ST5-MRSA-II) and Pediatric (ST5-MRSA-IV) clones.

It was shown that, different *SCCmec* types have been acquired by *S. aureus* strains with different genetic backgrounds, and this suggests that SCCmec was introduced several times into different *S. aureus* genetic lineages. ST8-MSSA in CC8 was shown to be the ancestor of the first MRSA strain isolated, that is, ST250-MRSA-I, with ST250 differing from ST8 by a point mutation in the yqiL gene. ST8-MSSA is a common cause of epidemic MSSA disease, and has acquired *SCCmec* types I, II and IV [88].

Another clone that is related closely to ST250 is ST247-MRSA-I, that is, the Iberian clone. These STs differ from each other by a single point mutation at the *gmk* locus. ST247-MRSA-I is one of the major MRSA clones isolated currently in European hospitals [84], and major ST within CC8 is ST239-MRSAIII, which corresponds to the Brazilian clone [86]. This clone has evolved by the transfer, through homologous recombination, of a 557-kb fragment of the chromosome of ST30 into ST8-MRSA-III.

Furthermore, MLST analyses showed that some of the first vancomycin-intermediate *S. aureus* isolates have emerged from ST5-MRSAII, a pandemic MRSA clone known as the NewYork/Japan clone [87, 88]. It has also been shown that multiple lineages of *S. aureus* harbor different *SCCmec* types among hospitalized patients in Australia [89].

Enright et al. in their study found that MRSA has emerged at least 20 times following acquisition of *SCCmec*, and that the acquisition of *SCCmec* by MSSA was fourfold more common than the replacement of one SCCmec with another. Interestingly, *SCCmec* type IV was found in twice as many MRSA clones as other *SCCmec* types, suggesting that most clones arise by acquisition of *SCCmec* type IV by *S. aureus* [90]. This is probably a result of the smaller size of *SCCmec* type IV compared with other *SCCmec* types, which may facilitate transfer of the cassette among staphylococcal species [98]. Furthermore, it has been shown that MRSA strains that belong to the major CCs (1, 5, 8, 22, 30, 45) are easier to transform with *mec*A-expressing plasmids tan are strains belonging to minor CCs. This indicates that the genetic background of *S. aureus* may be important for the stability of *SCCmec* [4, 91].

The population structure of MSSA is genetically more diverse than that of MRSA, and that MRSA originated from a limited number of epidemic MSSA lineages through transfer of the *SCCmec* [92, 93]. It was shown that CC5, 22, 30 and 45 were all derived from epidemic MSSA lineages that have acquired *SCCmec*, since they differed from each other, and from ST8, at six or seven loci [90]. This suggests that some MSSA genetic backgrounds may not provide a stable genetic environment for *SCCmec* integration.

6. Epidemiology of methicillin-resistant *S. aureus*

MRSA first appeared among hospital isolates of UK in 1961 [94] corresponded to *SCCmec* I and it was a typical representative of the archaic clone that rapidly spread in European

countries [4]. These strains, described as epidemic MRSA (EMRSA or HA-MRSA) spread gradually throughout most hospitals all over the world [83, 84, 90]. In the 1970s, MRSA isolates appeared in the USA, Australia and Japan.

In 1982, MRSA *SCCmec* type II was discovered in Japan, and the new York/Japan clone, to which it belongs, also spread, after which the isolation of the MRSA strain *SCCmec* type III followed, in New Zealand [4, 84, 88]. In Asian countries, two epidemic clones, the Brazilian clone (sequence type 239 [ST239]-MRSA-IIIA) and the New York/Japan clone (ST5-MRSA-II) have been found to be prevalent and to possess unique geographic distributions [95]. In central Europe, a close relative of the well-described ST5 MRSA clone, namely ST225, as prevalent in health care setting [54, 90]. This spread from Europe to the USA [54]. In Africa the presence of the following clones: sequence type (ST) 5-MRSA-I, ST239-MRSA-III, ST612-MRSA-IV, ST36-MRSA-II and ST22-MRSA-IV have been reported [97, 98]. ST239 is also common in mainland Asia, South America and parts of Eastern Europe [54]. In the genomes of 63 globally distributed ST239 isolates, SNPs with highly similar sequences between strains from Portugal and South America, which is suggestive of the historical and modern links between these two regions were identified [91].

The particularity of the population structure of MRSA isolations in Latin America was the predominance of only two clones, the Brazilian clone (CC8-ST239-*SCCmec*III) in the strains from Brazil, Argentina, Chile and Uruguay and the Chilean/Cordovan clone [99, 100].

HA-MRSA is mainly multi-resistant, and the choice of antibiotics for treating infections caused by hospital-acquired MRSA is limited to vancomycin and linezolid and mainly causes serious infections in patients who are predisposed in some way: those with a weak immune system, after long-term hospitalization, long-term use of antibiotics, a progressive underlying illness, etc. infection by MRSA strains in hospital conditions is usually preceded by colonization of differing duration [88].

In the 1990s, a new type of MRSA appeared in the USA causing infections in the community among healthy and younger people who had no history of hospital admission or medical treatment in the previous year was reported in Western Australia [70]. These types of MRSA strains were described as CA-MRSA [85, 93].

HA-MRSA strains are genetically distinct to CA-MRSA [101]. Particularly, CA-MRSA strains are usually sensitive to antibiotics other than β-lactams and contain staphylococcal and carry a smaller version of the genetic region responsible for methicillin resistance (*SCCmec* IV or *SCCmec* V), and often produce the Panton-Valentine leukocidin (PVL) [74, 75].

CA-MRSA strains in the USA are most commonly in a genetic cluster designated as PFGE type USA300, MLST type ST8 or spa type t008 [93]. The clonal complexes determined in the SARM-AC strains correspond to CC1 (ST1-SARM-IV) circulating in Asia, Europe and USA, the CC30 (ST30-SARM-IV), CC8 (ST239-SARM-III/IV) detected in Australia, Europe and South America and the USA300 (ST8-SARMI-IV) with a wide geographic distribution which includes countries in Europe and Latin America and in the USA. Also the ST59 in Asia and the USA and the ST80 in Asia, Europe and the Middle East [84, 95, 96, 100]. A variant of clone CC30 (EMRSA-16/ST36-MRSA-II) that is prevalent in the UK and the clone CC5 (ST125-SARM-IV) circulating specifically in Spain exists [50]. Throughout Europe, the CA-MRSA strain is CC80:ST80-IV is the most predominant [83, 84].

The information gathered from MLST indicates that MRSA has evolved multiple times, leading to the circulation and predominance of particular clonal complexes and sequence types [55]. In the case of *SCCmec* type IV, CA-MRSA is an element smaller than the other elements, appears more genetically mobile and does not, at present, carrying additional antimicrobial resistance genes is presented [70]. It also appears to occur in a more diverse range of MSSA genetic backgrounds, suggesting that it has been heterologously transferred more readily from other staphylococcal species [54, 101, 102].

Oosthuysen et al. found a high PVL prevalence, especially among MSSA clones [98]. The MSSA population identified and studied could act as a potential reservoir for CA-MRSA clones upon the acquisition of *SCCmec* elements, leading to the rise of PVL-positive CA-MRSA clones [75, 98].

With the studies of molecular typing in *S. aureus,* they have managed to establish the structural differences between the bacteria isolates and the dynamics of dissemination and the characteristics of the isolates in an outbreak.

Molecular epidemiology studies in MRSA show the predominance of number small clones around the world, that is, they have a capacity for dissemination pandemic, probably favored by cross infections with strains closely related between hospitals from faraway places.

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Chapter 3

Dairy *Staphylococcus aureus*: Epidemiology, Drug Susceptibilities, Drug Modulation, and Preventive Measures

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Additional information is available at the end of the chapter

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Abstract

Staphylococcus aureus is an emerging pathogen from dairy animals' mammary glands. Among various risk factors associated with this pathogen are unhygienic milking procedures, improper preventive techniques, and lack of germicidal teat dipping before and after milking. Methicillin-resistant *S. aureus*, coagulase positive *S. aureus*, vancomycin-resistant *S. aureus*, and biofilm-producing *S. aureus* are common strains of *S. aureus* being isolated from dairy milk these days. They have huge economic and public health concerns. Trials of antibiotic susceptibility proposed variable responses, while drug modulation and drug synergistic proved to be hope for its treatment. Some of the plant derivative, phages, and nanoparticles are non-antibiotic sources to treat *S. aureus.* Various attempts to treat *S. aureus* at the world level have been carried out but require more researches to be undertaken in order to prevent it. The chapter concludes that *S. aureus* from dairy needs equal attention as is given to *S. aureus* from the human origin, and researches are required to probe solutions.

Keywords: *Staphylococcus aureus*, prevalence, public health, antibiotic susceptibility, prevention strategies

1. Introduction

Staphylococcus aureus is a Gram-positive coccus, non-motile, non-spore-forming, catalase positive, coagulase positive, and facultative anaerobic bacteria that is responsible for all kinds of mastitis in dairy animals. The pathogen has developed the capability to resist action of most of the antibiotics used in disease management. The inflammation in mammary

glands of dairy animals is a worldwide issue, origin of which may be infectious or non-infectious. The latter is less frequent that, however, occurs due to physical insult to mammary glands during or after milking. The bacterial contaminants cover most of the part of the infectious causes of mastitis. The pathogenic pattern of *S. aureus* involves adherence to mammary epithelial cells and to the extracellular components. Subsequent to this comes the mammary epithelial invasion where they remain in membrane-bound vacuoles of the mammary gland's epithelial cells. The phagocytic activity of the phagosome is bypassed to induce apoptosis. The recurrent subclinical infections occur because of bacteria dwelling in epithelial cells in that they inflict injury there by the endocytic process. Not only are the economic and health challenges limited to bovine, but potential zoonosis exists due to *S. aureus*. A clonal complex 398 representative of livestock-associated methicillin-resistant Staphylococcus aureus (MRSA) has proven the ability of colonization and serious health consequences in humans who are in close contact with animals.

2. Prevalence of *Staphylococcus aureus* from dairy milk

2.1. Cattle and buffalo milk

Bovine mastitis has been reported with more than 140 bacterial species in addition to minor prevalence owned by fungi, algae, and virus where *S. aureus* stands in an average number as the first causative agent for this malady. *S. aureus* prevalence is variable, starting from less than 10% to as high as 65%. The staphylococcal isolates from the bovine subclinical mastitis have been tuned to 85% in Pakistan. Recent studies in Canada reported a 46% *S. aureus* prevalence at herd level. The pathogen is invariably present in both buffalo and cattle but some of the studies report higher prevalence in buffalo than cattle. Variation in prevalence of *Staphylococcus aureus* within and among different dairy species might be because of bacterial survival in keratin layer of mammary glands where various immune evasive techniques like biofilm production are the reasons for lower shedding of bacteria from the mammary gland's environment. Other factors include geographic area variation, breed, specie, and farm management. The prevalence of mastitis in buffalo is higher than that of cattle in various studies. The fact behind might be higher nutritive values of its milk that favor growth of bacteria. The longer teats with pendulous shape also support bacterial invasion which is comparatively higher than that of cattle [1]. Some of salient features for spread of this pathogen are regarded as Milker's hands, flies, and towels spread these pathogenic bacteria to clean udders during milking practices.

2.2. Camel milk

Studies about camel diseases reported lower prevalence of mastitis before the twentieth century. The reason for not prioritizing camel mastitis was that higher contents of lactoferrin are recognized as antibacterial. However, later studies identified various aspects of mastitis. The studies on the microbial involvement find *S. aureus* invariably present with various percentages. Its prevalence has been noted as lowest as 1.8% in Saudi Arabia and as high as 83% from Kenya. Pakistan has reported 74.04% of *S. aureus* prevalence in the camel community from the desert. The majority of studies reported a non-comparable higher prevalence of *S. aureus*.

However, some of the studies report it to be second major pathogen after *Streptococcus agalactiae,* thus, meaning that the prevalence of this pathogen was noted to be 20.35% at the world camel community so far. Variation in prevalence has been attributed to the irregular shedding pattern of this bacteria, different hygienic standards at farms, unhygienic milking process, and lower than required inoculum (0.1 mL) for streaking on growth media and biofilm production. Unhygienic conditions are dominant in the desert environment which results in heifer-harboring intra-mammary bacteria that upon giving birth keep shedding in milk. Use of devices to stop calf suckling, tick infestation, udder deformities inflicted by thorny bushes, and camel pox favors the spread of mastitis. All these factors are unleashing *S. aureus* incidences. Some diagnostic screening techniques have been attempted for early identification of this pathogen that otherwise requires biochemical protocols. Sensitivity of the California mastitis test is reported to be 68% in a study. In case of camel milk, the California mastitis test is difficult to perform in that large numbers of cellular fragments surrounded by plasma membrane having rough endoplasmic reticulum and mitochondria but lacking nucleus are found normally in milk. Presence of these cellular fragments creates false positive results that normally require lymphocyte, neutrophils, and macrophages that are markers of inflammation.

2.3. Goat milk and sheep milk

S. aureus prevalence in caprine milk has been tuned to 66% [2]. Raw milk cheese and unpasteurized milk is consumed on traditional grounds. Apart from quality and quantity of milk deterioration, bulk milk contamination with *S. aureus* reflects the severity of farm's subclinical and clinical mastitis. This could be a reason of high observation of *S. aureus* from bulk milk. Swiss dairy farms reported 30% of goat and sheep herds having been identified with *S. aureus*. The virulence of *S. aureus* observed was same both for caprine and for ovine in terms of *splE* and *sdrD*. Higher prevalence of *spLE* was observed in goat milk while *lukM* was observed in sheep milk. Genes that code superantigen-like proteins (*ssl*) were observed to be immunoevasive as they interfere the toll-like receptor system. The biofilm-forming gene (Q7A4X2) was observed in addition to *sdrD*, *splE*, and *lukM* that are mainly virulent factors of *S. aureus* isolated from small ruminants. Studies have reported that the *Staphaurex* latex agglutination test is a more effective diagnostic tool in case of caprine and ovine *S. aureus*. This test was reported to present 51% of results as false negative when used as a diagnostic test for bovine *S. aureus*. Information is limited on raw bulk milk contamination with *S. aureus*. There are limited studies reporting ewe's bulk milk tank contamination with *Staphylococcus aureus*. The heterogeneity in *S. aureus* reporting exists with peak percentage during the 2003 studies that reports 33.3% [3, 4]. However, meat of ewes is reported to have 20–94% of *S. aureus* incidences [5].

2.4. Risk factors

The animals in older age are more prone to mammary gland infection due to dilated teats, previous repeated exposure to infection, and lower immune response [6]. The animals in old age are at double the risk with mastitis than animals of younger age. On the other hand, some of the studies did not find age as a risk factor for mastitis. The unhygienic conditions at farms along with other risk factors may result in infection to animals irrespective of age. Lactating animals are more prone to Staphylococcal infection because at lactation state spread of contagious pathogen increases if hygienic measures are not adopted. The periparturition period

is most susceptible to disease because of lower immune response during this period. Some of the studies report higher prevalence of disease in late lactation with reasons of lower immune response [7]. Early lactation was also found susceptible in some of the studies with reasons of higher milk production which is positively correlated with spread of mastitis. Ticks work to spread the pathogen from one animal to other. They create a suitable environment to aid microbial pathogenesis. Most of the studies have reported higher prevalence of mastitis in cases where ticks were infecting.

The higher parity was found more susceptible to infection. This was justifiable with carryover of infection from one parity to the next. Some of the researchers did not find the correlation of mastitis with parity number [8]. *S. aureus* being contagious is positively correlated with an unhygienic milking system. Fore milking stripping is found with *S. aureus* that may spread to other animals if hygiene is not adopted [9]. While conducting studies on prevalence of *S. aureus*, it is advised to discard the first few strippings of milk. However, spread of environmental mastitogens is not linked with fore milking stripping. The farms where teat dipping before and after milking with chlorhexidine and iodine is being practiced are reported to have reduced chances of disease [10]. They are discovering alter resistance of antibiotics against foodborne bacteria [11].

2.5. Types of *S. aureus* strains isolated from dairy milk

Staphylococcus aureus comes from the family Staphylococcaceae and genus *Staphylococcus*. The *Staphylococcus* genera is reported to have 42 species that are further categorized based on coagulase production. There are some of species of this genus that are normal inhabitants of the skin and mucus membrane. The species other than *Staphylococcus aureus* that produce coagulase and are found in etiologies of mastitis include *Staphylococcus intermedius* and *Staphylococcus hyicus*. The production of coagulase may not be strictly adherent to these strains due to advent of genetic variation. In addition to this phenotypic identification, results' interpretations exist [12]. This invites nucleic acid target-based techniques for the sake of identification and classification. The virulent genes namely spa igG binding, icaA, icaD, agrI-agrIII, cap. fnbA, fnB, hla, hlb, clfA, nuc, and spa X-region are linked to bovine mastitis. Added to these are *mecA* gene, *blaZ* gene, vancomycin-resistant genes, and hyper-virulent genes that increase diagnostic labor [13]. Salient virulence factors that include hemolysin (alpha, beta, gamma, delta), heat-shock protein, enzymes (nuclease, lipases, protease, staphylokinase, esterase), capsular polysaccharides, slime, cell-adhered proteins (fibronectin-binding protein, elastin-binding protein, collagen binding protein, and protein A) have been frequently identified from dairy milk. They have direct effects on public health (14, 13). Methicillin-resistant *S. aureus* not only spreads to animals but also has been reported to develop outbreak in humans [14].

Biofilm-producing strains in subclinical and clinical mastitis are also one the rise. These are sessile microbial-derived community of cells that get attached to substrate or to the each other whereby they are embedded in self-produced extracellular polymeric substance of diverse constituents like DNA, protein, carbohydrate and so on [15]. Identification of these strains from *S. aureus* has been tuned to 61%, and this may increase in environment where suitable risk factors are observed. The intra-mammary infections settled for long periods call for adhesive colonies' aggregation that are surrounded by the self-created exopolysaccharide matrix, the biofilm. The biofilms evade phagocytosis because of higher size. The matrix of biofilm varies from specie to

specie, and also the environmental circumstances play a role in determining the complexity of the biofilm's matrix. Biofilms have proven resistance to ultraviolet light, antibacterial drugs, biocides, biodegradability, and amplified genomic diversity, diversified degradability, and higher production of secondary metabolites [16]. The resistance to antibiotics is attributed to the physical barrier (exopolysaccharide), limited growth of bacteria in biofilm, accumulation of antibiotic-degrading enzymes in the matrix, and transformation of protein in the cell wall of bacteria.

2.6. Public health concern

Staphylococcal food poisoning (SFP) cases have been reported by Centers for Disease Control (CDCs) in the USA to be as high as 240,000 [17], while Europe observed 386 outbreaks in 2014 (Anonymous, 2015). The outbreaks are characterized with diarrhea and violent vomiting soon after ingesting SFP food. Analysis realized the involvement of enterotoxins and super antigens; some of those were classical enterotoxins like SEA-SEE and others were newly identified [18]. The necessitation of identification of *S. aureus* from domestic animals is impartial because of their residency in animals that act as a reservoir for onward infection. The feature is in addition to their role in compromised livestock economy [19]. The spread to public health presented new strains entitled LA-MRSA (livestock-associated methicillin-resistant *Staphylococcus aureus*). The frequent isolation of LA-MRSA has been observed by farmers, veterinarians, and farm workers' family members [20]. *S. aureus* produces heat-resistant enterotoxins that are one of the leading food poisoning causes. They are actually of 26900–29600 Da, molecular weight moiety, that up till the moment is nearly 20 different kinds of isolated entitled as staphylococcal enterotoxins (SE) and staphylococcal enterotoxin-like proteins (SEI). The prevalence of enterotoxins is rising in various dairies. These enterotoxins may be effective in milk even when *S. aureus* is not viable [21]. In Turkey, 46.9% of SEs of one or more types were isolated from subclinical bovine mastitis [22]. The Samsun province of Turkey presented 75% enterotoxins from raw milk [23], while 68.4% of strains isolated from bovine raw and pasteurized milk were positive for SE genes. The toxic proteins of bacteria exploit host tissues to produce nutrients for their growth. Staphylococcal enterotoxins are hypothesized to induce emesis. They are associated with inflammatory mediators like prostaglandin E_2, 5-hydroxyeicosatetraenoic acid, and leukotriene B4. The observed areas of inflammation in gastrointestinal tract appear with upper part involving stomach and intestine. The observable pathogenesis includes exudate in duodenum.

Not only had the raw but processed milk also reflected 10.4% of *S. aureus* prevalence, the analysis isolated five virulent genes encoding Paton-Valentine leukocidin, staphylococcal enterotoxin, toxic-shock syndrome toxin-1, methicillin resistance, and exfoliative toxin. More than 60% of strains presented greater than one virulent factor. The strains show variable response to various classes of antibiotics and even to the members of each class. Cheese made of goat milk may have this pathogen as some of the studies have detected 9.5% of this pathogen's involvement that was characteristically enterotoxigenic, coagulase positive, and methicillin resistant. The studies found six new alleles (glpf-500, pta-440, aroe-552, aroe-553, yqil-482, and yqil-496) and five newer sequence types (STs) that is to say ST 3431, ST 3440, ST 3444, ST 3445, and ST 3461 in *S. aureus* from goat milk. Isolation of novel alleles in *Staph areus* from goat is thought normal than those of bovine and humans in that more focused studies are scarce in case of goats.

2.7. Economic damages

Economic damages that are outcomes of clinical and subclinical mastitis are entitled as reduced milk yield, spoiled milk, lower milk quality, unstable taste, reduced milk processing, lower shelf life, and decreased yield of milk products. The ancillary economic burden includes treatment costs, spread of disease, culling, veterinarian fee, and labor costs. For staphylococci, losses to dairy in the Dutch dairy system were noted to be €293 per cow clinical mastitis. Dairy cattle per cow clinical cases were anchored to estimated €277 for the first three month's post-calving and €168 onward to the end of lactation. In US dairy circumstances, the estimated economic damages in dollars are estimated to be $1.8 billion/9 million dairy cows on an annual basis, exclusive of antibiotic residual in human diet, costs used to control milk's nutritive quality, and degradation of milk.

2.8. Drug susceptibilities and drug modulation

2.8.1. Susceptibility

The susceptibility of *S. aureus* from bovine mastitis is variable in the increase or decrease in resistance against antibiotics. Somewhere, *S. aureus* is noted to be pan-susceptible to antibiotics in studies from goats, while pan-resistance from bovine milk is also on record. The report of a retrospective study concludes two times the reduction of *S. aureus* resistance against penicillin while six times resistance against erythromycin over a period of 6 years [24]. This was not true in reports encompassing results of studies conducted in other geographical locations where resistance to the antibacterial drug increased to double of what was reported 12 years ago [1, 25]. The studies later to 2001, however, mention increase in general resistance of *S. aureus* strains against antibiotics. The difference in trends is attributed to evolution of resistance against local microflora being under therapy selection, traditions of farmers, drug regulation of country, local antibiotic therapy protocols, and number of processed samples in the study. Bacteria use horizontal gene transfer from resistant to sensitive strains [26]. The prevalent resistance genes noted in *S. aureus* encode for oxacillin (mecA), erythromycin (ermA, ermB, erm C), gentamicin (aac-6/aph-2), and tetracycline (tetK and tetM), penicillin (blaZ), and vancomycin [27].

Penicillin and cephalosporin group of antibiotics are found to be generally resistant against *Staphylococcus aureus* from bovine and camel milk. However, susceptibility varies from species to specie, region to region, strains of *Staphylococcus aureus,* and frequent exposure to antibiotics. Cefoxitin- and vancomycin-resistant strains are emerging. Linezolid is however effective in current dates against *Staphylococcus aureus* strains of bovine milk. The antibiotic trials have presented ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, chloramphenicol and tetracycline effective against *Staphylococcus aureus* that originates from various dairy animals. Higher susceptibility of *S. aureus* could be because of infrequent use of antibiotics in that area. Pan-susceptibility is noted higher than all dairy animals in that the drugs that usually face resistance by *S. aureus* of other dairy animals are quite effective in case of ovine *S. aureus*. Penicillin resistance is extensively noted while limited resistance was found when tested against *S. aureus* of ovine milk. The current status of ovine-based *S. aureus* was 100% susceptible at Greece farms, which thus reflects an absence of methicillin-resistant strains. The feature is attributed to very low pressure of antibiotic use at sheep farms in Greece. Traditional

farming is mostly on organic farming so they are safe from MRSA infection that in turn draws attention toward ovine milk as safe food.

2.8.2. Drug combinations

The increased resistance has been noted against all kinds of antimicrobials and no introduction of any new drugs has invited the use of newer drug combinations. Some of the drugs from the aminoglycoside group are although effective but reportedly linked to ototoxic and nephrotoxic effects due to their continued use. The drug combination requires antibiotics to target at different sites. Penicillin group in conjunction with aminoglycoside was reported as potent, effective, and safe. Combination of cephalosporin (cefaroxil) and penicillin (amoxicillin) showed synergistic effects against 80% of resistant isolates. Within the drug class, for example, β-lactam with ß-lactam combinations, efficient results were presented as well [28], and in vivo trials have also proved their effectiveness. Aminoglycosides are potent drugs that create fissures in the outer portion of bacterial cell wall by binding with 30S ribosomal subunit, thus misreading mRNA. Penicillin in combination with chloramphenicol has been reported synergistic in some of the studies while antagonistic results have also been reported. Antagonism reported in some studies claim penicillin to activate while chloramphenicol to deactivate murein hydrolase that in its function is responsible for lysis of bacteria. The general concept describes bactericidal and bacteriostatic to be antagonistic which is now true in other studies [29]. This trend might be because of diversification of genetic variation in modern pathogens.

2.8.3. Plant derivative effects/drug modulation

Plants have various antimicrobial peptides like c-thionin and thionin Thi 2.1 tested against intracellular *S. aureus* of bovine mastitis. These peptides in addition to their antibacterial activity work as immune modulators. The extracts from other plants like ethanolic extract of propolis (EEP), a resinous mixture obtained by honeybees from plants, are reported to be highly biologically active against *S. aureus* mastitis. There is limitation attached with this in terms of lower minimum inhibitory concentration (MIC) when tested in milk environment. However, authors have suggested its in vivo activity against mastitis. Monolaurin, a coconut oil derivative made of glycerol monoester of lauric acid, has also presented antibacterial activity against *S. aureus*. Extracts of *Tabernaemontana divaricata* (L.) have shown significant efficacy against a group of microorganisms of bovine mastitis origin which demand further research to be undertaken [30]. The bovine clinical mastitis-based *S. aureus* showed sensitivity against crude extracts of *Combertum molle* and *Commicarpus pedenculosus* medicinal plants [7].

The development of resistance demands some alternative ways to combat *S. aureus*. The bacterial resistance takes place due to impairment in binding as a consequence of genetic mutations, enzyme production, for example, hydrolyzing that impaired amide bond, and efflux extrusion which is responsible for reduction in drug concentration inside the cell [31, 32]. The constituents of plant extracts modulate resistant mechanism techniques of bacteria to the extent where they become sensitive. Various in vitro trials have been reported with promising results against multidrug-resistant *S. aureus*. Some of the plants naturally growing in animal-rearing areas are featured with antimicrobial characteristics. A few among 500 plant species are explored in documentation with proven antibacterial effects. There is wider scope yet to

be explored as an alternative source of bactericidal. *Calotropis procera* and *Eucalyptus globolus* have proven activity against *S. aureus*. These plants are salt and drought resistant growing in wider quantities in the surroundings of animal-rearing far areas. Plant extracts in synergy with antibacterial drugs target various sites of *S. aureus,* thus modifying phenotypic resistance to sensitivity [33]. The antibacterial activity is attributed to flavonoids, alkaloids, saponins, glycosides, phenols, and tannins. The active ingredient gives rise to the porous cell wall, thus releasing contents from cytoplasm, electron-transport chain inhibition, and interference with sphingolipid inhibition [34]. The activity may vary depending on the variation in solvents for extraction, the stage of plant's cultivation, geographical area, method of extraction, and specific mode of action [32].

2.8.4. Nanoparticles

The recent few years have presented nanoparticles (NPs) which have emerged as a cost-effective potential antibacterial against various pathogens. Nanoparticles (NPs) are small particles of 1–100 mm size and work by disruption of cell membrane, simultaneous activation of multiple mechanisms, and action as antibiotic carriers. They break physical barriers made of biofilms to reach bacterial cells embedded inside whereby antibiotics cannot reach alone. Oxidative stress, non-oxidative stress, and metal-ion release mechanisms are used by Ag, Mg, NO, ZnO, CuO, Cu_2O, Fe_2O_3, FeO, and many others to kill bacteria. Multiple drug-resistant *S. aureus* showed a 177 mm zone of inhibition at 80 μL of silver nanoparticles. Nitric oxide nanoparticles are not only effective against *S. aureus* but also play a role in prevention of mastitis in dairy animals. They alone and in combination with antibiotic preparation are evaluated in vitro targeting *S. aureus* and also the wound healing. Nanoparticles that work as drug delivery include liposomal NPs, inorganic NPs, polymer-based NPs, terpenoid-based NPs, and polymer micelle NPs. These nanoparticles coat antibiotics and effectively reach to the site where the drug mechanism does not work. The encapsulation of antibiotics with nanoparticles makes drugs express their potential that in alone are unable to impart their effect. Tilmicosin-solid lipid and amoxicillin are sometimes unable to deliver their effects alone but encapsulation with nanoparticles complements their activity at full bloom. Hydrogel-coated nanoparticles, for example, sliver hydrogel coated, proved to be superior in antibacterial activity, viscosity, and drug release. Several studies have proven their efficacy in terms of wound healing, normal skin appearance, and hair growth. These particles help make production of hydrogen per oxide and reactive oxygen species at wound site that help cure infection/mastitis. The small-size particles confer cell death and reduction of bacterial resistance.

2.8.5. Other alternatives

Phages are alternative sources where no other therapeutic action against pathogens is workable. The staphylococcal species may effectively be lysed with phage K. Moreover, phage K can be used prophylactically against intra-mammary infections endorsed by *S. aureus*. Phage K is reported as a pocket rocket against mastitis by some researchers. On the other hands, phages are vulnerable to mammary glands' immune system and whey protein of milk that render phages ineffective [35]. Studies are needed to rule out pharmacokinetics and pharmacodynamics in addition to the challenges of their administration into tissues. Another polyvalent

virulent phage, MSA6, is isolated from cow mastitis that is being used as a potential universal anti-staphylococcal agent [36]. This particular phage is applicable against a wider host range, superior lytic action, and importantly are thermo stable. The peptidase derived from the bacteriophage, $CHAP_K$, of cow mastitis is effective both at prophylactic and at therapeutic ends. Biofilm-producing strains of *S. aureus* may be effectively prevented from biofilm production and disruption of already established biofilms. Stress can affect bacteriophage activity. Some bacteriophages including Sabp-P1, Sabp-P2, and Sabp-P3 are resistant to environmental stress [13]. Apart from limitations, phages resistant to stress can be best applicable for futuristic staphylococcal mastitis treatment.

Cytokines are proteins with a definitive role in cell signaling. Some of the recombinant cytokines of bovine origin like IL-2, IFn-c, and TNF-alpha stimulate both kinds of immunity (innate and acquired) in mammary glands. However, their effect in combination with antibiotic therapy is additive against mastitis [37]. Beta-lactoglobulin protein is normally present in mammal's whey while lactoferrin is present in milk, bronchial mucus, saliva, and tears. Both molecules have proven activity against *S. aureus*-based mastitis. These proteins complement a higher spectrum of antimicrobial activity either applied alone, in combination with each other, or in combination with antibiotics. There are other animal-derived sources like marine sponges that exhibit antibacterial activity against a wider range of Staphylococcal species when used in extracts. These sponges include species from Cinachyrella, Haliclona, and Petromica that were effective antimicrobial agents against 61% of tested microorganisms [38].

2.8.6. Bacteria with probiotics

Mechanisms of persistence of *S. aureus* in intra-mammary environments still need to be explored but evasion of host immune system and adherence to epithelial cells of mammary glands are some of the known in this regard [39]. Some bacteria like *Weisella confuse* and *Lactobacillus casei* are reported to produce certain compounds that are active against internalized persistence of *S. aureus*. Lactic acid bacteria have the ability of adherence to epithelial cells, thus resisting *S. aureus* pathogenicity by its competitive adhesion ability, production of H_2O_2 , competition in nutrition utilization, and host immune modulation [40]. Continuous use of Weissella strains and their metabolites are reported to be effective alternatives of antibiotics in control and prevention of mastitis [41].

2.9. Prevention strategies against dairy *S. aureus*

Controlling *S. aureus* in dairy products is needful for commercial and profitable small-scale cow farming for improving milk quality to consumers as well as dairy industries. Although a significant progress has been done in over the last 30 years, *S. aureus* seems to be still severe in dairy animals around the world. The lack of effectiveness of the current strategies (principally based on antiseptic teat dipping after milking and antibiotic therapy during the dry period) to suppress *S. aureus* has promoted in the sense of vaccine preparation against *S. aureus* which is a reasonable/alternative approach for the control of these microorganisms associated with mastitis. Studies have reported higher prevalence coupled with increased resistance to antibiotics in *S. aureus* isolates of camel mastitis [42, 43]. The emergence of discrepancies in resistance

identification has also added to increased resistance in terms of unjustified use of antibiotics to combat *S. aureus* [44, 45]. Resistance to antibiotics and the phagocytosis phenomenon leads to treatment failure against *S. aureus,* so the vaccine development against mastitis is an exigent to prevent new infections by *S. aureus* for commercial dairy farms. Anti-*Staphylococcus aureus* vaccines give different results, depending on the type of vaccine, the adjuvant used, and some other factors involved.

2.9.1. Vaccinal targets in S. aureus

Several studies have shown that different soluble and cytotoxic factors are involved which increase the *S. aureus* pathogenicity by using different pathogenic factors, for example, pseudocapsules, toxins, clumping factors, protein A, and fibronectin-binding protein. It has been suggested that these pathogenic factors should be considered for preparing mastitis vaccine to be used in field conditions. Furthermore, it has recently been suggested that the *S. aureus* vaccine may be much effective if it is multicomponent integrated with surface proteins, toxins, and surface polysaccharides. Recently, it has been proposed that more than 99% of the world's bacteria exist as biofilm producers. Experts at disease control and prevention centers, the USA, estimate that 65% of human bacterial infections are involved in biofilm production [46]. The term "biofilm" for bacteria refers to a structured population of bacterial cells enclosed in a self-produced polymeric matrix and attached to an inert or living surface that forms a protected growth pattern that allows surviving in harsh environments. Biofilm-forming microorganisms produce a particular mechanism to attach the surface to form a microbial community, producing a three-dimensional structure of mature biofilms [47]. Their growth rate, composition, and resistance to anti-biocides, antibiotics, and antibodies are all different because they up-regulate and/or down-regulate about 40% of the genes. This makes it difficult to eliminate the infections due to such microorganisms with therapeutic doses of antimicrobials. A better understanding of the mechanisms by which they can evolve and survive in sessile environments can help in designing control strategies against *S. aureus* [48].

2.9.2. Vaccines in action

There is growing evidence that *S. aureus* can form biofilms in the udder of dairy cows affected by mastitis. Biofilms not only affect the host's immune system but also prevent the action of antibacterial drugs, leading to persistent infection. *S. aureus* causes chronic infections, resulting in significant financial losses in most of the cases [49]. Biofilm is an important factor in the virulence of *S. aureus* [50]. It has been demonstrated that the active immunization of exopolysaccharides extracted with strongly adherent *S. aureus* isolates provokes the defensive immunity against mastitis [51]. The use of antibiotics to treat and prevent *Staphylococcus aureus* mastitis has driven mastitis researchers in preventing udder infections through vaccine due to high costs, low cure rates, high antibiotic resistance, and consumer concerns about antibiotic residues in milk and meat [52]. Various mastitis vaccines have been studied including inactivated whole cell, live vaccines, cell wall components, bacterin toxoid, and antigen extracts with or without adjuvants. Findings of some researchers are summarized later. Israeli workers [53] supervised a large number of field trials with commercially available vaccine (MSTIVAC

I; Patent No. PTC/IL 98/00627) for *S. aureus* mastitis. The authors observed a 42–54% reduction in first and second lactation in SCCs and 0.5 Kg/day/animal increase in milk production as compared to unvaccinated (control) cows. In the vaccinated group, only 3 out of 228 animals (1.3%) while in the control group 6 out of 224 (2.7%) was detected. No statistical analysis was conducted as these numbers were low for statistical analysis between vaccinated animals and non-vaccinated (control) animals. Later on, findings of Athar [54] at the Department of Clinical Medicine and Surgery of the University of Agricultural Faisalabad (UAF), Pakistan, confirmed that the locally developed polyvalent vaccine for mastitis (incorporated with killed *S. aureus*, *S. agalactiae* and various *E. coli*) provided protection for new infections as well as eliminated existing infections in dairy buffaloes. Similarly, other authors have also observed such findings with locally prepared *S. aureus* vaccines (plain bacterin, oil-adjuvant bacterin, live attenuated vaccine and dextran sulfate-adjuvant bacterin) [47]. Brouillette et al. [55] conducted a DNA immunization study against the *Staphylococcus aureus* aggregation factor A (CIF-A). It has been found that preincubation of *S. aureus* with serum obtained from vaccinated mice reduces the ability of pathogens to bind up to 92% of fibrinogen. These preincubated bacteria were phagocytosed by elevated macrophages in vitro, whereas, in in vivo trials, these were less toxic when evaluated experimentally in a mouse-mastitis model. However, DNA-immunized mice could not resist the challenges caused by the intraperitoneal route. The results showed that DNA immunization can be used as a new method to prevent *S. aureus* infection.

2.9.3. Current scenario of vaccines

In this new era, mastitis has been one of the imperative diseases in dairy cows, despite tremendous advances in improving overall udder health. Epidemiological studies have showed a lot of variations in biological cure rates (from 0 to 80%) following antibiotic treatment, but these do not show the significant loss of antibiotic activity of the major classes. Repeated infections often lead to the formation of biofilms in bacteria. In the case of microorganisms, biofilm formation is caused by subsequent physiological and significant genetic changes resulting in loss of sensitivity to antibiotics, thus leading to development of resistance to antibiotics of different classes. Ahmad and Muhammad [56] conducted a study on the preparation and evaluation of *S. aureus* and *S. agalactiae* aluminum hydroxide adjuvant mastitis vaccine in rabbits. Biocharacterization of both bacteria was done from 95 milk samples collected aseptically from mastitic buffalos. Immunogenicity, pathogenicity and susceptibility testing of antibiotics was performed. Bivalent aluminum hydroxide adjuvant vaccine was developed in the Mastitis Research Laboratory at Clinical Medicine and Surgery Department, University of Agriculture Faisalabad-Pakistan. The vaccine was proved stable, sterile, and safe to use. Rabbits were used to evaluate the quality of the vaccine and the antibody response. For this purpose, rabbits were divided into two (GA and GB) groups, having 10 rabbits in each. Rabbits in the GA group were injected with *S. aureus* and *S. agalactiae* aluminum hydroxide-adjuvant mastitis vaccine, while the rabbits in second group (GB) remained non-vaccinated. To check the antibody titers in rabbits of group GA, indirect hemagglutination inhibition assay (IHA) was performed. GA rabbits had the highest anti-*S. aureus* serum antibody titer (GMT) which was 78.8 at the 45th day, dropping slightly to 73.3 on day 60 post-vaccination. IHA titer gradually increased for *S. agalactiae* at days 45 and 60 after the inoculation of vaccine. The cumulative mean antibody

titer (CMT) for the vaccinal *S. aureus* was 44.94 and CMT for the vaccinal *S. agalactiae* was 46.56 as compared to the control group. The CMT was significantly higher in vaccinated group at days 45 and 60 after the vaccination than the control group. The study showed that the bivalent aluminum hydroxide-adjuvant vaccine was immunogenic in rabbits. To evaluate the *S. aureus* bacterin, Middleton [57] used a lactating cow model to study the ability of this vaccine to prevent intra-mammary infections (IMI) of staphylococcal (*S. aureus* and coagulase-negative staphylococci (CNS)). Assessment parameters were the vaccination effects on somatic cell count (SCC) and the effects of vaccine on the antibody isotype of milk. For this purpose, 90 lactating cows of Holstein-Friesian were selected and divided into two groups. One group (n-44) served as vaccinated group and the second group (n-46) was the control group. First group received 5 mL of bacterin vaccine, 2 shots, 14 days apart. Milk samples were collected from individual quarters for bacterial culture before each shot and then collected monthly for 6 months. For determining IgG1, IgG2, IgM, IgA, and SCC, composite samples of milk were collected on days 0, 14, 28, 49, and 70. The authors did not observe any new IMI in any group and this was not different significantly between the groups ($p > 0.05$) of mammary quarter infection. The vaccine in herds having been reported with coagulase-negative staphylococcal prevalence (30%) and *S. aureus* prevalence (3%) in intra-mammary infection did not respond well to newer *Staphylococcal* infections. Another study has evaluated a multicomponent vaccine to eradicate staphylococcal biofilm infections [58]. Selected antigens including glucosaminidase (hypothetical conserved protein), an ABC transporter lipoprotein, and conserved lipoproteins have been found in previous studies to sustain and up-regulate expression in biofilms both in vitro and in vivo. For these antigens, the antibody was first used in a microscopic study to determine its expression in an in vitro biofilm. In biofilms, each of the four antigens exhibits heterologous production at different locations within a complex biofilm community. The four antigens were delivered simultaneously as a quadrivalent vaccine. As vaccine antigens were specific for biofilms, antibiotic treatments were also used to remove residual and non-adhered planktonic cells. The results showed that the clinical and radiographic symptoms were reduced to 67 and 82%, respectively, when the vaccine was given with vancomycin treated in biofilm rabbit models with chronic osteomyelitis. It was compared with animals infected or not treated with vancomycin. In contrast, only vaccination resulted in a modest and insignificant reduction.

Recently, Raza [47] evaluated the role of a bacterin toxoid prepared from a strong biofilm-producing *S. aureus* in effective immunization of rabbits. The strong biofilm-producing *S. aureus* selected from 64 isolates of staphylococci was used to prepare bacterin toxoid, and aluminum hydroxide gel was added as an adjuvant. The vaccine was evaluated in rabbits by challenge protection assay and humoral immune response. The mortality rates in control and vaccinated groups were 80% and 10% at day 7 post-challenge and 100 and 20% at day 15 post-challenge, respectively. Serum antibody titer (GMT) was significantly higher (294.0) in vaccinated group as compared to the control group rabbits (2.63) at day 45. The results showed an increased antibody production in the vaccinated group that was capable of preventing establishment of new *S. aureus* infection in rabbits as compared to the control group. Based on the results of the present study, a short-term clinical trial was conducted in dairy cows and buffaloes which also showed effectiveness of vaccine as indicated by a significant difference in the prevalence and incidence of mastitis, high level of variation in the microbiological examination of milk, reduced

intra-mammary infections, and somatic cell counts between vaccinated and control groups of dairy cows and buffaloes.

3. Conclusions

Staphylococcus aureus from dairy animal origin has obtained more serious attention than that of human origin in terms of pathogenicity, strain variability, response to antibiotics, public health concern, and economic losses to the dairy industry. Apart from bovines, camel and caprine are noted with surged prevalence since last few years. Being contagious in nature, *S. aureus* has been found to be emerging due to the increase in the span of risk factors. As there is an increase in antibiotic resistance against *S. aureus*, the hope in the form of non-antibiotics like nanoparticles, plant derivatives, bacterial, and phage based-remedies exists. Vaccines, as a preventive strategy, have been implemented at local and commercial levels. The research is required for a comprehensive approach both at preventive and at therapeutic levels.

Conflict of interest

Authors declare no conflict of interest

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Chapter 4

Prevalence, Diagnosis and Local Susceptibility of Staphylococci Infections

Funmilola Abidemi Ayeni

Additional information is available at the end of the chapter

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Abstract

Staphylococci are normally harmless commensals occurring on the skin, mucous membrane and the general environment. However, they are increasingly implicated in different infectious states. Of particular interest is the advent of methicillin-resistant *Staphylococcus aureus* (MRSA) with its attendance resistance to beta lactam antibiotics. Several infectious states are now emerging with staphylococci being implicated in the infections, e.g. *S. saprophyticus* has been implicated in urogenital infection. It would be interesting to document the prevalence of staphylococci in different infectious state. The identification of staphylococci is supposed to be a straightforward procedure, but an alarming misidentification rate is emerging in low resource laboratories, especially in places where identification is solely by growth and fermentation on mannitol salt agar (MSA). Finally, empirical treatment of any staphylococci infection will depend on local suspectibility pattern of the strains as the susceptibilities vary from environment to environment. This chapter summarizes the current knowledge regarding the prevalence, diagnosis and local susceptibility of staphylococci in different parts of the world.

Keywords: misdiagnosis, prevalence, susceptibility, staphylococci, antibiotics, identification

1. Introduction

Staphylococci have long history and association with mankind. From their presence in amniotic fluid, all through to adulthood, they were once regarded as harmless commensals with beneficial effects, e.g. by competing with pathogenic bacteria, but they are now implicated in life-threatening infections. Coagulase-negative staphylococci (CoNS) cause invasive infections in some vulnerable groups of patients, e.g. immunocompromised patients, preterm

neonates and people with indwelling medical devices [1]. *Staphylococcus epidermidis* is observed in 33% of blood samples collected from neonates, and it is the second most prevalent species observed in orthopedic device-related infections [2, 3]. *S. aureus* and *S. epidermidis* were ranked first in opportunistic infections and the major causative agent of medical implants and nosocomial infections in developing countries [4]. *S. epidermidis* was the commonest infectious species followed by *S. saprophyticus* and *S. haemolyticus* in a clinical study [5]. *S. lugdunensis* is implicated in infectious endocarditis. Although CoNS possess lesser virulence properties than *S. aureus*, they are more challenging due to their large proportion of methicillin-resistant strains with increasing numbers of isolates resistant to glycopeptides [6].

Most *S. aureus* infections used to be in the healthcare setting, but they are now established as a causative agent of serious infections in the community [7]. Immunocompromised individuals are at higher risk of general *S. aureus* infections (particularly invasive infection, e.g., bacteremia) than immunocompetent individuals. The emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) has led to an increase in the severity of infections of CA-MRSA in the last two decades [8]. Also, prevalence of methicillin-resistant coagulase-negative staphylococci has been reported, e.g. prevalence of nasal and pharyngeal carriage of methicillin-resistant *S. scuri* among 195 inpatients and healthcare workers in an healthcare center in Serbia has been reported as high in hospitals and possible dissemination across hospital wards can occur [9]. Several factors are responsible for transmission of *S. aureus* infections, e.g. domesticated animals in household transmission [7], neonates and breastfeeding mothers, immunocompromised patients, use of an indwelling intravascular plastic catheter, surgical incisions, open wounds, or burns.

The main aim of this chapter therefore is to highlight the current knowledge on prevalence, diagnosis and local susceptibility of staphylococci in different parts of the world.

2. Prevalence of staphylococci infections

S. aureus causes many infections including skin, soft tissue and invasive infections (complicated pneumonia, bacteremia, musculoskeletal infections and endocarditis). The common staphylococci infections are otitis media, bacteremia, skin infections, pneumonia, endocarditis, neonatal infections, osteomyelitis, food poisoning, toxic shock syndrome and scalded skin syndrome. However, there are increasing reports of staphylococcal infections in other parts of the body and vulnerable population.

2.1. Prevalence of staphylococci in different diseased condition

There are emerging facts on the role of staphylococci in central nervous system infections. In a multinational study performed with 2583 patients in 37 referral centers in 20 countries to understand the burden of community-acquired central nervous system infections between 2012 and 2014 [10], staphylococci and *Listeria* were responsible for frequent infections in immunocompromised patients. In another study of 102 patients on maintenance

hemodialysis, of 1402 patients hospitalized for infectious spondylodiscitis over a 13-year period, MRSA was the commonest pathogen found in the infectious sites followed by coagulase-negative staphylococci [11].

The role of staphylococci in burns and skin infections is well documented. In a retrospective study of 123 patients hospitalized in the burn center of Marrakech over a period of 3 years (2013–2016), there were 103 infections per 1000 days of treatment in different infective sites (blood (18%), skin (69%), lungs (1%) and urinary tract (12%)) with the main infectious organisms being: *Staphylococcus* sp. (37.7%) and MRSA in 22% of cases [12]. In Japan, a recent study investigated the antimicrobial resistance in pathogens isolated from skin and soft-tissue infections (SSTI) at 40 dermatology departments and clinics resulting in isolation of three main organisms (579 of *S. aureus* 141 (MRSA 24.4%), 240 of coagulase-negative staphylococci and 41 of *Streptococcus pyogenes*) identified from 860 strains [13].

Staphylococci are frequently implicated in hospital-acquired bacteremia especially those associated with intravascular catheters and staphylococcal bacteremia and they are important cause of morbidity. A study that described the epidemiology of healthcare-associated bloodstream infections for 71,039 patients in 338 Polish hospitals between 2012 and 2015 found that the most frequently isolated microorganisms were staphylococci (45.6%) and most of them were coagulase-negative (64.4%) and usually caused catheter-related infections. Of 53 *S. aureus* isolated, 24.5% were methicillin-resistant [14].

Immunocompromised patients are at higher risk of staphylococcal infections. In a study over a period of 1 year of *S. aureus* colonization, 81% of adults with human immunodeficiency virus (HIV) have higher rates of colonization than the general population [15]. Children with immunocompromising conditions are very vulnerable to *S. aureus* infections with the higher risk of development of complications in children with malignancy and high rates of resistance to antimicrobials [16].

Staphylococci are frequently implicated in neonatal infections usually within 6 week after birth with diseased conditions such as skin lesions, pneumonia, bacteremia, meningitis. In an epidemiology study of neonatal infection from 2005 to 2014 in 30 UK neonatal units, *E. coli* (15%), *S. aureus* (14%) and CoNS were prominent causes of late-onset sepsis in the neonates [17]. In sub-Saharan Africa, there is limited information on large-scale study on prevalence of staphylococci infections. However, Seale et al. [18] reported that from all neonatal admissions in a local hospital in Kenya from 1998 to 2013 to determine CoNS in neonates, CoNS was isolated from blood culture in 995 of 9552 (10%) neonates and the neonates with CoNS have higher risk of convulsions. Staphylococci were the most prevalent organism in a hospital-based case–control study in the Regional Hospital, Cameroon between September 2015 and August 2016 [19].

Otitis media is an inflammation of middle ear which may lead to hearing loss. *Streptococcus pneumoniae, Moraxella catarrhalis, Haemophilus influenzae* and *S. aureus* are some of the organisms implicated in acute otitis media. To assess the frequency of bacterial agents in 185 chronic suppurative otitis media, *Staphylococci* spp. (64.9%) were the most prevalent bacteria observed [20]. In some developing countries, otitis media may prevail as a result of illiteracy, poverty

and poor hygiene. In a study of 263 pus samples from 240 patients in a developing country, highest incidence of otitis media was observed in 1–10 year age group with the commonest bacteria isolated being *S. aureus* (36.11%) and CoNS (8.08%) [21].

2.2. MRSA prevalence in Africa

There is variable information on prevalence of MRSA in Africa. The prevalence was lower than 50% in most African countries and higher prevalence since 2000 has been observed in many African countries (except South Africa). In South Africa, the prevalence decreased between 2006 and 2011, while it varied between 23 and 44% for 2000–2007 in Botswana. It increased from 16 to 41% between 2002 and 2007 in Tunisia; in Libya, MRSA prevalence was 31% in 2007, while in Egypt and Algeria the prevalence was 45 and 52% between 2003 and 2005, respectively, while northern Nigerian had higher MRSA prevalence than the southern part with 55 and 39% prevalence in Ethiopia and Ivory Coast, respectively [22].

In a review of 34 reports from 15 countries in Africa, CC5 is the predominant clonal complex in healthcare setting in Africa. Hospital-associated MRSA was identified in nine African countries with limited spread of European ST80-IV clone to Algeria, Egypt and Tunisia and lack of distinct difference between MRSA responsible for hospital and community infections. However, the community clones (ST8-IV and ST88-IV) were observed in the hospital and community settings in Madagascar, Angola, Príncipe, Cameroon, Ghana, Gabon, Nigeria and São Tomé [23].

The overall prevalence of MRSA was 22.6% from 142 *S. aureus* isolates obtained from 261 samples sourced from university staff, students and fomites in Awka, Nigeria with the carriage rate being higher in females than male and highest in individuals of 20–30 years [24]. In a neonatal septicemia study involving 202 infants with risk factors for clinical features of septicemia in the first 3 days of life, 12.5% culture were positive with the predominant organisms being *S. aureus* (52%) and 30.7% being MRSA [25]. In intensive care unit of a Nigerian hospital, out of 71 patients with healthcare-associated infection, bloodstream and urinary tract infections were the commonest infections, and *S. aureus* was the commonest cause of bloodstream infection with 80% of the *S. aureus* being MRSA [26]. In surgical site infections for 103 patients with orthopedic surgery in a hospital in Port Harcourt, Nigeria, the commonest pathogen was *S. aureus* (34%) including 15 patients with MRSA [27]. and prevalence of MRSA with *mecA* gene as 42.3% from 156 *S. aureus* in clinical isolates from South Western Nigeria with domination of SCCmec II and SCCmec V [28].

A significant decline in antibiotic resistance was observed in Northeastern Nigeria in contrast to the worldwide trend of increasing resistance rates as stated in a study involving changes in population structure of *S. aureus* isolates in 2007 and 2012 from Northeastern Nigeria with a reduction in resistance to erythromycin, penicillin, clindamycin and gentamicin in 2012 with a decrease of MRSA [29]. The authors have confirmed low to moderate prevalence of MRSA in Nigeria in various studies. Ayeni et al. [30] reported 0.5% MRSA prevalence in nares of healthy adults. In another of our study by Ayeni et al. [31] where prevalence of MRSA in samples analyzed in Medical Microbiology Unit of University College Hospital, Ibadan between May and October 2012 was done. A 50 *S. aureus* strains were obtained with 34% of the studied *S. aureus* strains being phenotypically identified as MRSA strain.

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3. Diagnosis of staphylococci infections

Identification of staphylococci is supposed to be a simple straightforward procedure that involves culturing of clinical specimens or pure bacterial strains on Columbia agar, mannitol salt agar (MSA) or tryptic soy blood agar. If pure biochemical identification is to be used, then Gram-positive, nonmotile, non-spore-forming, facultative anaerobic cocci occurring mainly in clusters and catalase-positive strains are selected for further tests. Coagulase test will distinguish between *S. aureus* and coagulase-negative staphylococci in clinical specimens. There is a current interest in small colony variant (SCV) of staphylococci which are nonhemolytic, nonpigmented and characterized by pinpoint colonies about 10% of the size of the normal colonies [32]. The SCV of *S. aureus* can contribute to persistent infection and they are associated with increased antibiotic resistance.

3.1. Overview of different staphylococci identification methods

Rapid latex and hemagglutination assays allows presumptive identification of *S. aureus* based on the detection of clumping factor, capsule types 5 and 8, protein A. They have high sensitivity (98–100%) and lower specificity (72–99%) which may be as a result of false-positive reactions occurring with some CoNS strains [6]. Novobiocin resistance is routinely used to distinguish the intrinsically resistant *S. saprophyticus* subsp. *saprophyticus* from other CoNS, e.g. *S. epidermidis* group. Tube coagulase test with horse plasma has been stated as a very accurate method to differentiate between *S. aureus* and CoNS. It has a high specificity [30].

There are commercial and automated systems for identification of staphylococci, e.g. Staphylococcus-specialized API Staph, Vitek 2, Rapidec Staph and ID32 Staph strips (bioMérieux) system, BBL Phoenix automated microbiology system, Crystal identification system's Rapid Gram-Positive ID kit (BD, MD), Pos ID Panel family (Siemens, Deerfield, IL), Sherlock microbial identification system (MIDI, Newark, DE) and the Biolog systems (Biolog, Hayward, CA) [6].

There are also several molecular approaches for identification of staphylococci. Conserved regions with species-specific sequences of universally occurring genes are amplified, for differentiation at the species level, e.g. 16S and 23S rRNA, *gap, gyrA, sodA, rpoB* and *tuf* genes. Sequencing of 16S rRNA gene has wide application in identification of bacterial species. However, other genes have been observed to be superior to 16S gene for identification of staphylococci. In a previous study, the author had also confirmed previous knowledge that sequencing of *tuf* gene has more discriminatory power in identification of staphylococci than partial sequencing of 16S rRNA gene [33]. Also, partial sequencing of *rpoB* gene has better identification power than partial 16S rRNA gene sequencing for the differentiation of *Staphylococcus* subspecies [34].

SCV strains grow on blood agar as pinpoint colonies and they are often nonreactive in normal biochemical tests because their laboratory detection could be affected by their altered metabolism and long generation time. Therefore, molecular methods, such as amplification of species-specific DNA targets or 16S rRNA partial sequencing, become the method of choice for their identification [35].

Microarray-based diagnostics test may combine identification of staphylococci with detection of virulence factors and drug resistance in strains. In positive blood culture smears, a nucleic acid hybridization assay (*S. aureus*/CNS PNA FISH; AdvanDx) targeting rRNA gene sequences with the principle of nucleic acid fluorescence in situ hybridization (PNA FISH) can be used for rapid identification of *S. aureus* [6]. Furthermore, spectroscopic and spectrometric methods, e.g. Fourier transform infrared (FTIR), Raman spectroscopy and MALDI-TOF MS are currently used in diagnostic laboratories. MALDI TOF has become a universal quick and accurate method for identification of microorganisms including staphylococci, and the principle is based on spectra obtained by molecular weight for individual fragments.

Identification of MRSA is primarily by cefoxitin disk screen test, the latex agglutination test for PBP2a or selective chromogenic agars [30]. Commercial tests are also available for identification of MRSA with combined detection of *mecA* and toxin genes. By using a multiplex PCR approach, *mecA* and *femA* can be simultaneously detected for rapid identification of MRSA and to differentiate *S. aureus* (*femA*1) from CoNS especially in blood samples [36]. An excellent correlation was reported between the broth microdilution assay and detection of antibiotic resistance genes by multiplex PCR [37].

In some institutions, there is active surveillance that uses rapid laboratory techniques to evaluate nasal swab specimens and routinely screen admitted patients, e.g. high-risk patients, patients with previous MRSA infection, vascular, orthopedic, or cardiac surgery patients for MRSA.

3.2. Wrong identification of staphylococci

Due to lack of adequate resources, wrong identification of *S. aureus* which could lead to wrong diagnosis of *S. aureus* infections has been observed. Mannitol salt agar (MSA) is often used in many laboratories in some developing countries, e.g. Nigeria for identification of *S. aureus*. The initial design of the agar was with the claim that it supports the growth of coagulase positive staphylococci only by being a selective and differential medium: The composition is 7.5% sodium chloride, mannitol as the carbohydrate and phenol red. It was claimed that colonies of CoNS and other salt-tolerant organisms will produce pink or red colonies, while *S. aureus* will grow on MSA as yellow colonies [38]. However, there are several evidences to disprove this claim.

One hundred and eight-five isolates that had been previously isolated from the nares of college students' volunteers in Southern Nigeria were identified by various methods. Growth on MSA and slide coagulase tests was highly inaccurate for identification of *S. aureus* although it is an indication of staphylococci; however, this should be taken with caution because other organisms like *Brevibacterium* can also grow on MSA with yellow colonies [30, 39]. The study confirmed that tube coagulase test with horse plasma, MALDI TOF mass spectrometry and PCR amplification of the *spa* gene are accurate diagnostic methods for identification of *S. aureus*. Also chromogenic medium, chromIDTM MRSA plate (bioMérieux, France) and Slidex MRSA Detection Kit (bioMérieux, France) were accurate for detection of MRSA [30].

In another study by Ayeni and Odumosu [40], it was noted that some organisms are being wrongly identified as *S. aureus* in phenotypic identifications. The study evaluated inaccurate identification of other organisms as *S. aureus* by collecting 507 phenotypically identified

S. aureus strains (identified by Gram staining, characteristic growth and fermentation on mannitol salt agar and blood agar and coagulase formation) obtained from 8 states in Southern Nigeria. Standard identification of the isolates was done in the study by sequencing of 16S rRNA gene and detection of *spa* gene. Fifty-four (11%) of the total isolates were confirmed as *S. aureus,* while the rest were CoNS with 85% misidentification, *Bacillus* sp. with 12% misidentification and *Brevibacterium* sp. with 3% misidentification. The study reported an alarming rate of false positive identification of *S. aureus* which could have resultant effect of misdiagnosis and subsequent wrong antibiotic prescription especially in emergency situation. Therefore, we demonstrated that CoNS grows and ferments mannitol on MSA. Standard methods should be used for identification of *S. aureus*.

We also studied 171 strains of CoNS which have been previously identified as *S. aureus* as a result of growth on MSA. The strains were collected from different locations in Nigeria, and ViTEK 2, MALDI-TOF MS and partial sequencing of 16S rRNA gene sequencing (gold standard) were used for identification. It was discovered that all strains (13 species of CoNS) grow on MSA and ferment mannitol. All tested strains of *S. warneri, S. epidermidis, S. pasteuri, S. sciuri, S. nepalensis, S. xylosus, S. capitis* and *S. haemolyticus* were correctly identified by MALDI-TOF, while all strains of *S. gallinarum* and *S. kloosii* were misidentified by MALDI TOF with total absence of *S. gallinarum* in the MALDI-TOF database at the period of this study. All tested strains of *S. warneri, S. epidermidis, S. xylosus S. gallinarum, S. sciuri, S. capitis* and *S. haemolyticus* were correctly identified by ViTEK, while the equipment misidentified *S. pasteuri* and *S. nepalensis*. It was concluded that growth on MSA for *S. aureus* is the same with CoNS and therefore the growth media cannot differentiate between CoNS and *S. aureus*. ViTEK seems more accurate than MALDI-TOF in identification of CoNS [33].

4. Susceptibility of staphylococci to antibiotics

As a basic principle, empiric use of antimicrobials should be guided by local epidemiology and antimicrobial susceptibility pattern as well as the clinical state of the patient, with final therapy determined by culture and sensitivity data. Vancomycin is the drug of choice for the treatment of MRSA infections, while clindamycin is the commonly used antimicrobial for CA-MRSA infections. However, many strains are emerging with reduced susceptibility to vancomycin for *S. aureus* and CoNS strains. MRSA are resistant to penicillin but susceptible to penicillinase-stable penicillins, such as methicillin and oxacillin. Healthcare-associated MRSAs are multiple resistant to other commonly used antimicrobial agents, including fluoroquinolones, erythromycin, tetracycline and clindamycin, while community-associated MRSAs are often resistant only to β-lactam agents and sometimes erythromycin and fluoroquinolones. In a study, 80% resistance to ampicillin was observed in CoNS, while resistance to cefoxitin and ceftriaxone was observed in 58% of the isolates [5].

In an antibiotic susceptibility study, 75.9% sensitivity to rifampicin, 100% sensitivity to vancomycin and linezolid was reported in catheter-related bloodstream infections in 58 (20 *S. aureus* and 38 CoNS) staphylococci in an Egyptian tertiary hospital with the recommendation

that linezolid and rifampicin could be used effectively against MRSA isolated from catheter-related bloodstream infections [41].

In another study on molecular epidemiology of trimethoprim resistance in 598 human *S. aureus* isolates collected in different locations across sub-Saharan Africa [Gabon, Nigeria (two), Namibia and Tanzania] [42]. About 54% of strains were observed to be resistant to trimethoprim and the resistance mostly mediated by *dfrG* gene, which is widespread in Africa. The study discourages the use of the drug for the treatment of SSTI caused by CA-MRSA.

Susceptibility of *S. aureus* strains to linezolid, rifampicin, teicoplanin, vancomycin, mupirocin phosphomycin, fusidic acid, daptomycin and tigecycline with 55 and 72% resistance to tetracycline and trimethoprim/sulphamethoxazole, respectively, has been reported, while in another study involving *S. aureus* isolates obtained from infection and asymptomatic carriers in Lagos and Ogun States, Nigeria, higher resistance was observed for aminoglycosides in clinical isolates, and more prevalent resistances to quinolones and tetracycline were observed in carrier isolates [37, 43].

Daptomycin and quinupristin/dalfopristin have been proposed as an alternative to glycopeptides in the treatment of MRSA infections, while the use of telithromycin is discouraged [44]. Also, all MRSAs were sensitive to amikacin, ciprofloxacin and chloramphenicol, while all methicillin-sensitive *S. aureus* were sensitive to ampicillin/sulbactam in a study [25], while fusidic acid resistance was reported in 93.7% of isolates, from prevalence of nasal and pharyngeal carriage of MRSA among inpatients and healthcare workers in an healthcare center in Serbia [9].

Osteomyelitis occurs more frequently in children, causing pains, chills and fever. Osteomyelitis regularly involves prolonged systemic antibiotic use, and dalbavancin, linezolid and vancomycin were active against staphylococci implicated in bone and joint infections [45].

All *S. aureus* strains in otitis media case in a developing country were sensitive to gentamycin [21], while ciprofloxacin was stated as the most effective antibiotic for treatment of bacterial chronic suppurative otitis media [20]. In a study on the prevalence and antimicrobial susceptibility pattern of external ocular bacterial infections in Ethiopia, the prevalence of MRSA infection was 24%, and multidrug resistance was observed in 87% of the isolated bacteria [46].

Ayeni et al. [30] reported susceptible to fusidic acid, rifampicin clindamycin, vancomycin and linezolid, with observed high resistance to penicillin and trimethoprim in 185 staphylococci, which had been previously isolated from the nares of college students' volunteers in Southern Nigeria. In another study by Ayeni et al. [31] where the current resistant pattern of *S. aureus* to β lactam antibiotics in samples analyzed in Medical Microbiology Unit of University College Hospital, Ibadan between May and October 2012 were evaluated. A 50 *S. aureus* strains were obtained which were highly resistant to erythromycin (72%), clindamycin (78%), aztreonam (70%) and amoxycillin (92%), but highly susceptible to imipenem (90%). Variable resistance was observed to cefotaxime (62%), ceftazidine (50%), cefoxitin (66%), ceftriazone (52%) and amoxycillin/clavulanic acid (50%). All the isolates resistant to amoxicillin/clavulanic were, however, susceptible to ≥1 of the cephalosporins. All phenotypic identified MRSAs were resistant to amoxicillin, erythromycin, clindamycin amoxicillin/clavulanic acid

and ≥1 cephalosporin (except 1). About 88% of the studied MRSA strains were sensitive to imipenem. *S. aureus* strains (42%) susceptible to amoxicillin/clavulanic acid were resistant to amoxicillin. A synergy was observed between imipenem and aztreonam in some isolates which were resistant to aztreonam but sensitive to imipenem which may be an indication that combined therapy of imipenem and aztreonam may result in enhanced antimicrobial activity of aztreonam. We concluded that cephalosporins are still relatively effective for treatment of *S. aureus* infections due to in vitro evidence.

Another study in our group determined antimicrobial resistance of staphylococci isolated from urogenital tracts of humans with a presumptive diagnosis of urinary tract infection in 45 urogenital samples (endocervical swab, high vaginal swab and urine) from outpatients at Igbinedion University Teaching Hospital between April and May 2010. Ten isolates (22% of the total samples) of staphylococci were obtained. All the isolates were multidrug resistant with exhibited resistance to ≥5 antimicrobials and 100% resistance to ciprofloxacin, nitrofurantoin, augmentin, ampicillin and ceftriazone. All CoNS strains were susceptible to doxycycline, while *S. aureus* strains were relatively susceptible to TMP/SMX [47].

Ceftobiprole and ceftaroline are new cephalosporins active against *S. aureus*, including MRSA strains causing infections like pneumonia and staphylococci soft tissue infections in adults. They have been recently approved in Europe (Ceftobiprole by the European Medicines Agency) and the USA (Ceftaroline by U.S. Food and Drug Administration) for treatment of *S. aureus* and MRSA infections. However, resistance to these antibiotics is emerging, and it is often associated with mutations in *mecA,* increasing in the production of PBP4, which mediates resistance to ceftobiprole and ceftaroline [48]. However, a global surveillance conducted prior to the European launch of ceftaroline revealed 4 *S. aureus* from 8037 tested strains with ceftaroline resistance [49]. In another study on ceftaroline against 1971 *S. aureus* isolates collected from seven countries in the Asia-Pacific region in 2012 [50], there was ceftaroline susceptibility rate of 86.9%, and surprisingly in Thailand, more than half (52.8%) of isolates were resistant to ceftaroline. Minimal resistance to ceftobiprole has been reported by Hodille et al. [51] (1 of 440 *S. aureus*) strains isolated from bronchopulmonary infections being resistant to ceftobiprole, while another study involving MRSA isolates from colonization (n = 37) and infection (n = 23) isolated from Côte d'Ivoire, Congo, Gabon and Nigeria, 16.7 and 15% of strains were resistant to ceftaroline and ceftobiprole, respectively, and surprisingly detected only in Nigeria [52].

4.1. Susceptibility of staphylococci to non-antibiotic substances

Other natural and beneficial bacteria have been found to be effective against staphylococci in vitro. This has been demonstrated in previous studies. The first discussion is on medicinal plants that have been proven over many generations to be effective against several infectious diseases. The plants from the genus *Combretum* have been shown to be part of recipe for the traditional treatment of various diseases with broad antimicrobial spectrum of 36 species of the genus having antimicrobial activities [53, 54]. In our study involving the antibacterial activities of the methanol extracts from the leaves of *Combretum hispidum, Combretum racemosum* and *Combretum platypterum* against seven strains of MRSA in vitro, extract from

Combretum racemosum leaves had high anti-MRSA activities (0.16–1.25 mg/mL MIC values) on all tested strains of MRSA [55]. This could be a potential source of newer antimicrobial agent against MRSA infections.

Lactic acid bacteria (LAB) are beneficial bacteria with good antimicrobial activities against many pathogenic bacteria. We reported good inhibition of growth of uropathogenic *S. saprophyticus* and *S. aureus by L. fermentum, L. brevis, L. plantarum, Streptococcus durans* and *Lactococcus lactis* [56]. In another study in our group, LAB from salad vegetables have anti-MRSA abilities in vitro against five confirmed MRSA strains. *P. pentosaceus* and *L. cellobiosus* exhibited widest zones of inhibition. In the agar overlay, *P. pentosaceus* and *W. confusa* showed the widest zones of 28 and 24 mm, respectively [57].

In a recently published co-culture study that we did with LAB and MRSA, the cell free supernatant of *L. fermentum* and *L. plantarum* was generally active against MRSA, the largest zone of inhibition was 13 mm with *L. plantarum*. Further experiment of co-culture was done in the study with MRSA and *L. plantarum* 9, *L. buchneri* SM04, *L. fermentum* 008, *L. brevis* 21 and *Weissella paramesenteroides*. All the tested LAB reduce the viable counts of MRSA from 10 log to 3 log after 24 h of co-incubation, while *L. plantarum* 9 and *L. fermentum* 008 totally inhibited the growth of MRSA after 72 h of co-culture and the MRSA could not grow in overnight culture of LAB, after 24 h of incubation [58]. In another study from our group, LAB strains were assayed for antimicrobial ability against two uropathogenic *S. aureus* strains. The selected *Staphylococcus* spp. was generally resistant to macrolides (100% resistance to clarithromycin), aminoglycosides (50–90%), fosfomycin and rifampicin (20%), while 70% resistance was observed in co-trimoxazole. Lesser resistance was observed in the quinolones (10–20%), and β lactams antibiotics have variable resistance (30–90%). However, 22 LAB strains had strong suppression of target *S. aureus* strains with clear zones (>10 mm) around the streaks [59].

5. Conclusion

Staphylococci are implicated in various infectious states in different parts of the world with high prevalence. However, characteristics growth on mannitol salt agar is insufficient to differentiate between *S. aureus* and coagulase-negative staphylococci. Other standard identification methods should be used. Staphylococci antibiotic susceptibility varies between different locations and site of infection.

Conflict of interest

The author declares that there is no conflict of interest.

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Chapter 5

Molecular Features of Virulence and Resistance Mechanisms in Nosocomial and Community-Acquired *Staphylococcus aureus*

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are known for their emergent multi-drug resistance phenotypes, implication in nosocomial infections and outbreaks worldwide, being commonly associated with hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) skin and soft tissue infections. *S. aureus* causes a wide spectrum of clinical symptoms, ranging from mild to life-threatening diseases; disease severity is determined by microorganism-related virulence factors and host condition. The ability of these strains to form microbial biofilms, one of the main pathogenicity factors, generates difficult medical problems, favored by the widespread use of large invasive medical procedures (probes, catheters, heart valves, prostheses). Contamination of these devices is associated with the risk of subsequent development of human infections. The knowledge of virulence and antibiotic resistance patterns of HA-MRSA and CA-MRSA and encoding genes are very important for supporting effective infection control measures and therapy of staphylococcal infections.

Keywords: *Staphylococcus aureus*, MRSA, resistance, virulence, biofilm

1. Introduction

Staphylococci are commensal bacteria that form part of microbiota of human and animal skin and mucous membranes. Among more than 40 species of the genus, *Staphylococcus aureus* is colonizing the nostrils and skin of ~30% of the population [1, 2]. *S. aureus* is an opportunistic pathogen,

causing infections when it crosses the barriers of natural defense and escapes the mechanisms of anti-infectious protection. Factors favoring staphylococcal infections include local (lesions of the skin, the presence of implants, catheters, etc.) and general (innate or acquired deficiencies of the immune system such as complement system deficiencies, granulocytopenia, agranulocytosis, AIDS, diabetes, immunosuppressive treatments, etc.). It is able to cause a plethora of community (CA) and health care (HA) infections, ranging from superficial skin infections to severe, and potentially fatal, invasive diseases [3–5] due to its ability to produce a spectrum of virulence factors and resistance to multiple antibiotics, frequently encoded by mobile genetic elements (MGEs) [6], which have eased the persistence of *S. aureus* in hospital environment [7]. *S. aureus* is causing skin and soft tissue infections (SSTIs), endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, foreign-body infections, and sepsis [8]. *S. aureus* is the most commonly isolated bacteria from wound infections and studies involving patients with chronic venous leg ulcers found *S. aureus* positive cultures in 88–93.5% of infections [9]. Bacteremia caused by *S. aureus* is associated with higher morbidity and mortality, compared with bacteremia caused by other pathogens, with an annual incidence rate of 20–50 cases/100,000 population, and a mortality rate of 10 and 30%. The highest mortality rates occur in patients with primary bacteremic pulmonary infections and infective endocarditis, whereas the lowest rates occur in patients with central or peripheral venous catheter-related infections [10]. The major concern refers mainly to methicillin-resistant *S. aureus* (MRSA) isolates. Health care-associated MRSA (HA-MRSA) are represented by the *S. aureus* strains isolated from patients after a hospitalization of two or more days or with the MRSA risk factors (history of recent hospitalization, surgery, dialysis, catheters, etc.). Community-associated MRSA (CA-MRSA) are those *S. aureus* isolates obtained from patients within 2 days of hospitalization and without the above-mentioned MRSA risk factors [11]. Infections by CA-MRSA isolates are usually associated with children, young age, recurrent infections and the use of injectable drugs [12].

In this chapter, we review some aspects related to resistance and virulence features in CA-MRSA and HA-MRSA strains, underlying the evolution of the highly successful community- and health care-associated lineages and their plasticity in ability to adapt to environmental changes.

2. *Staphylococcus aureus* resistance to antibiotics

Antistaphylococcal antibiotics are mainly targeting cell wall synthesis, proteins and nucleic acid synthesis, and different metabolic pathways. The large use of antibiotics, not only in the medical field but also in the agriculture has facilitated the evolution and spread of resistance genes [13]. Bacterial resistance can be constitutive (mutations of the target genes, efflux pumps overexpression, etc.) or acquired by horizontal gene transfer via various mobile genetic elements like plasmids, transposons, bacteriophages, pathogenicity islands, and staphylococcal cassette chromosomes [14]. Plasmids and staphylococcal cassette chromosomes in particular have played a central role in conferring resistance to β-lactam antibiotics and vancomycin [15].

Penicillin resistance is conferred by β-lactamase, an extracellular enzyme encoded by *blaZ* that is active when bacteria are exposed to β-lactam antibiotics. The enzyme acts on β-lactam ring by opening it through hydrolization [16].

Methicillin resistance requires the presence of the chromosomally localized *mecA* gene. The *mecA* gene and its regulatory elements, form the *mec* complex: SCC*mec* elements carry the *mecR1* and *mecI* genes, which regulate the expression of *mecA*, with increased *mecA* translation induced by β-lactam antibiotic exposure. SCC*mec* elements can also carry resistance genes for other antibiotics and heavy metals as well as the psm-mec locus, which encodes cytolysin termed phenol-soluble modulin-mec (PSM-mec) [16].

PBPs are membrane-bound enzymes that are used in cross-linkage of peptidoglycan chains by catalyzing the transpeptidation reaction [17]. PBP2a has a low affinity for β-lactam antibiotics and thus methicillin resistance also grants resistance to all β-lactam antibiotics [16].

Eleven types of SCC*mec* have been described, distinguished by the type of *ccr* gene complex that mediates the site-specific excision and insertion of the SCC*mec* cassette out of or into the bacterial genome and the class of *mec* complex that they bear [18].

There have been revealed molecular differences between CA-MRSA and HA-MRSA strains regarding the types of SCC*mec*: HA-MRSA strains carry the large staphylococcal chromosomal cassette mec (SCC*mec*) belonging to type I–III and containing the *mecA* gene, mostly universal among MRSA isolates and usually are resistant to several classes of non-β-lactam antibiotics. It seems that the large SCC*mec* types I–III are present in HA-MRSA strains and were transferred to *S. aureus* from a commensal staphylococcal species [19]. Carriage of the psm-mec locus from type II SCC*mec* elements attenuates virulence, suppresses colony spreading activity, reduces expression of the chromosomally encoded PSMa, and promotes biofilm formation [20].

HA-MRSA strains seldom carry the genes for the Panton-Valentine leukocidin (PVL). CA-MRSA isolates carry smaller SCC*mec* elements, most commonly SCC*mec* type IV or type V [21]. CA-MRSA strains are resistant to fewer non-β-lactam classes of antibiotics and frequently carry PVL genes. There has been suggested that the smaller SCC*mec* types IV, V, VI, and VII have been transferred to methicillin-susceptible backgrounds [21, 22]. One study suggested that the type IV SCC*mec* element has been transferred to an MSSA strain [23]. The type IV SCC*mec* was originally associated with MRSA infections in patients with no HA-MRSA risk factors [21].

There have been revealed that the deletion of the gene encoding PBP 4 in two common CA-MRSA isolates, USA300 and USA400, resulted in a 16-fold reduction in oxacillin and nafcillin resistance in these particular stains. These studies suggest that PBP 4 is a significant target for the discovery of agents effective against CA-MRSA [24].

There have been also reported CA-MRSA strains positive for *mec*A and PBP 2a that were phenotypically oxacillin susceptible [25]. It was therefore suggested that *mec*A expression alone does not appear to be sufficient to guarantee phenotypic methicillin resistance, and that the existence of additional molecular targets could be associated with the susceptibility of oxacillin in certain strains and the involvement of genes different from the known effectors of methicillin resistance in CA-MRSA. The *vraS*/*vraR* two-component regulatory system is required for oxacillin resistance in CA-MRSA [26, 27].

The emergence/re-emergence of successful *S. aureus* clones suggests a rapid bacterial adaption and evolution, which includes the emergence of antibiotic resistance and increased virulence and/or transmissibility.

Most of the nosocomial MRSA infections are caused by five major lineages that circulate internationally: CC5, CC8, CC22, CC45 and CC30 [28].

ST1 pulsotype USA 400—represented the most frequently CA-MRSA clone in the United States after 1990s; was characterized by carrying SCC*mec* type IV that has been usually susceptible to most non-β-lactam antibiotics and cause SSTIs. This strain lacked PVL genes and circulates in the community in Australia (WA-MRSA-1) and England [18, 29]. ST80 is PVL+-bearing SCC*mec* type IV and have been reported in several Western European countries such as Austria, Norway, Denmark, Sweden, England, Switzerland, Greece and France [18]. ST30 corresponds to phage type 80/81 nosocomial strain of *S. aureus* from United States during the 1950s and 1960s. These strains were MSSA strains that carried the PVL genes [30] and the SCC*mec* type IV element. ST59 strains are PVL+, they have diverse spa types and several SCC*mec* types were isolated from different countries worldwide like: Taiwan, Australia, Denmark, Netherlands England, and the United States [18]. In Taiwan, ST59 clones with a multidrug-resistant phenotype are the most encountered, having a distinctive SCC*mec* DNA sequence [31]. ST93 was first identified in 2000 in Queensland and New South Wales, Australia. It spread rapidly to become the predominant PVL+ MRSA clone isolated from infections in those regions. USA300 Strains has the following characteristics: the carriage of SCC*mec* type IV, PVL genes, and, in most strains, the ACME element. USA300 is classified as ST8 by MLST and is usually classified as t008 by *spa* typing. It is frequently susceptible to several non-β-lactam antibiotics and became the dominant CA-MRSA strains in the United States, has been also identified in Western Europe [18], Japan [32] and Australia, where it has been called WA-MRSA-12 [18, 33].

MRSA infections in the community can also be caused by livestock-associated MRSA (LA-MRSA). LA-MRSA is initially associated with livestock and differs from genotypic HA-MRSA and genotypic CA-MRSA in its genomic traits. CC9 clonal complex (LA-MRSA) is most frequent among livestock in Asia [34]. In the USA, ST5 (MRSA) was isolated from pigs [35]. CC1 (MRSA) is prevalent in Romanian nosocomial infections and has low host specificity [36].

The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV, which separates concatenated DNA strands. Resistance to quinolones results from the stepwise acquisition of chromosomal mutations [16]. Quinolone affinity is reduced by changes of the amino acids in the enzyme-DNA complex (quinolone resistance-determining region [QRDR]). The most common sites of resistance mutations are GyrA subunit in gyrase and ParC (GrlA in *S. aureus*) of topoisomerase IV. Drugs primarily target topoisomerase IV and the mutations at this level are essential for resistance [37, 38]. The confluence of high bacterial density, especially inside biofilms, the likely preexistence of resistant subpopulations, and the sometimes limited quinolone concentrations achieved at sites of staphylococcal infections creates an environment that fosters selection of resistant mutants [37].

An additional mechanism of resistance in *S. aureus* is induction of the NorA multidrug-resistance efflux pump with increased expression can result in low-level quinolone resistance [39].

S. aureus response to vancomycin inhibitory activity divides the strains into sensitive, intermediate and resistant. Vancomycin intermediate-resistant *S. aureus* (VISA) have MICs to vancomycin of 8–16 μg/ml (MIC = 4–8 μg/ml). There has been identified the existence of a pre-VISA stage of resistance known as heterogeneous VISA (hVISA) [16]. An hVISA phenotype

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refers to a mixed cell population—derived originally from a single colony of *S. aureus*—in which the majority of cells have little or no resistance to vancomycin (MIC ≤ 2 μg/ml) and a sub-population of cells is resistant to the antibiotic at the level of VISA (MIC ≥ 4 μg/ml) [40].

The molecular mechanisms that underlie development of hVISA are incompletely defined. Fundamental characteristics of the VISA phenotype include increased cell wall thickness, caused by differentially regulated cell wall biosynthesis and stimulatory pathways [15, 41], reduced cross-linking of peptidoglycan, decreased autolytic activity of the enzymes responsible to cell-wall turnover [15, 42], altered surface protein profile, dysfunction of the *agr* system and changes to growth characteristics [15, 43].

Molecular basis of the VISA phenotype is not fully understood but several genes/mutations are known to contribute to its development. The mutations within genes encoding two-component regulatory systems, such as *graRS* and *walKR* are of particular significance. GraRS differentially regulates transcription of cell wall biosynthesis genes and has been associated with a broad array of genes and regulators that play a role in the intermediate resistance phenotype [44]; GraRS also up-regulates genes in the capsule biosynthesis operon, leading to increased capsule production [44]. GraRS up-regulates the *dlt* operon and the *mprF/fmtC* genes, which are linked to teichoic acid alanylation and alteration of cell wall charge [15]. Point mutations within *graRS* reduced susceptibility to vancomycin [15, 45] and *graRS* mutations are linked to modified expression of global regulators, *rot* and *agr* [15, 44]. *rpoB* a gene encoding the DNA-dependent RNA polymerase β-subunit is commonly associated with increased resistance to vancomycin, prolonged propagation time and increased cell wall thickness [15, 46]. VISA isolates have been shown to have non-silent mutations in *vraSR*. Such mutations could lead to downstream up-regulation of over 40 cell wall synthesis genes, including genes required for producing cell wall derivatives such as D-Ala-D-Ala [15].

The VISA strains produce considerable amounts of peptidoglycan and this generates thicker, irregularly shaped cell walls. They also expose more D-Ala-D-Ala residues available to bind and trap vancomycin which acts as a further impediment to drug molecules reaching their target on the cytoplasmic membrane [16].

Complete vancomycin resistance in *S. aureus* (MIC ≥16 μg/ml) is conferred by the vanA operon (containing *vanA*, *vanH*, *vanX*, *vanS*, *vanR*, *vanY* and *vanZ* genes) encoded on transposon Tn1546, originally a part of a vancomycin-resistant enterococci (VRE) conjugative plasmid [15, 47]. The *vanA* operon is controlled via a two-component sensor-regulator system encoded by *vanS* and *vanR* that sense vancomycin and activate transcription of the operon, respectively, VanA, VanH and VanX together are essential for the vancomycin resistance phenotype. *S. aureus* can acquire enterococcal plasmids during discrete conjugation events. Vancomycin resistance in *S. aureus* is maintained by retaining an original enterococcal plasmid or by a transposition of Tn1546 from the VRE plasmid into a staphylococcal resident plasmid [15, 48].

3. *Staphylococcus aureus* adhesion and biofilm development

The broad range of infections caused by *S. aureus* is related to a number of virulence factors that allow it to adhere to surface, invade or avoid the immune system and cause harmful toxic effects to the host [49].

The initialization of the colonization process is started by the attachment of *S. aureus* to the host cell surface through adhesins. Proteins covalently anchored to cell peptidoglycans represent one major class of *S. aureus* adhesins, which attach to the extracellular matrix or plasma components via a threonine residue and are known as the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) [49].

Staphylococcal cell wall-anchored (CWA) proteins are secreted by the Sec system and share a C-terminal cell wall anchoring motif, hydrophobic domain and positively charged domain [50]. Foster et al. [50] proposed to classify the Staphylococcal CWA proteins into four groups based on structural motifs: MSCRAMMs (microbial surface component recognizing adhesive matrix molecules), the NEAT motif family, the three-helical bundle family and the G5-E repeat family. All of these types of CWA proteins are involved in staphylococcal biofilm formation. MSCRAMMs are adhesins that contain at least two IgG-like folds and employ a ligand binding mechanism called dock, lock and latch [50]. MSCRAMMS are composed of a binding domain, a cell wall spanning domain and a domain for the covalent or non-covalent attachment. These adhesins can bind one or more human proteins (collagen—mostly via Cna, fibronectin—via FnbAB, fibrinogen—via ClfAB and Fib) [51, 52].

The Staphylococcal MSCRAMMs are the Clf-Sdr family proteins, including bone sialoprotein-binding protein (Bbp), the fibronectin-binding proteins (FnBPs) and collagen adhesion (CNA) [53]. The Clf-Sdr family consists of Clumping factor A (ClfA), clumping factor B (ClfB) and the Sdr proteins. ClfA and ClfB are fibrinogen-binding proteins in *S. aureus* [50, 53]. Rot and agr affect bacterial binding to fibrinogen by regulating clfB but not clfA [54]. *S. aureus* has two fibronectin-binding proteins, FnBPA and FnBPB, encoded by fnbA and fnbB, respectively. FnBP binding to fibronectin induces bacterial invasion into epithelial cells, endothelial cells and keratinocytes [53]. The FnBPs affect biofilm formation by a self-association mechanism that is distinct from ligand binding and virulence [55, 56].

The NEAT motif family consists of the iron-regulated surface determinant (Isd) proteins who bind heme or hemoglobin, facilitating its transport into the bacterial cell, and they are up-regulated in iron-limiting conditions [57]. *S. aureus* IsdA is the most abundant CWA protein in iron starvation conditions, and also decreases surface hydrophobicity, which makes *S. aureus* more resistant to bactericidal fatty acids and peptides in human skin. IsdA also is able to bind human fibrinogen and fibronectin [53].

The sole three-helical bundle cell wall-anchored protein is Staphylococcal Protein A (SpA), which is present in all strains of *S. aureus*. SpA allows immune evasion by binding to the conserved Fc region of immunoglobulin IgG, and contributes to disruption of the host immune response by promoting bacterial survival in human blood after being released from the cell wall [53, 58].

G5-E Repeat Family: Aap/SasG—G5-E repeats are found in cell wall-anchored adhesins in Gram-positive organisms (are named after the five conserved glycine residues in each repeat). *S. aureus* SasG promotes attachment to human desquamated nasal epithelial cells via it's A domain [59]. Multiple studies have shown that the G5-E repeats of SasG and Aap are able to dimerize by binding to Zn^{2+}, forming a "twisted rope" structure [53, 60]. This property is thought to enable intercellular adhesion when adjacent SasG or Aap proteins dimerize via their G5-E domains.

Uncategorized CWA Proteins—the remaining uncategorized cell wall-anchored proteins are Bap and several Sas proteins, including SasA/SraP. SasX is another cell wall-anchored adhesin that has been shown to play an important role in virulence [53].

Surface-associated proteinaceous adhesins—autolysins AtlA and AtlE are found in *S. aureus* involved in cell wall turnover, cell division and cell lysis [53], they attach to extracellular matrix materials and can augment the biofilm matrix with eDNA by inducing cell lysis.

Non-proteinaceous surface-associated adhesins—wall teichoic acids and lipoteichoic acids have been shown to play a role in adhesion, colonization of host cells and biofilm formation. Wall teichoic acids are covalently linked to the peptidoglycan and consist of alternating phosphate and ribitol, while lipoteichoic acids attach to the outer leaflet of the cell membrane and have alternating phosphate and glycerol [61].

The polysaccharide intercellular adhesion (PIA) is a secreted polysaccharide that is synthesized by the *ica* operon and has been thoroughly studied in the context of biofilm formation, immune evasion, and pathogenesis. PIA is a glycan of β-1,6-linked 2-acetamido-2-deoxy-D-glucopyranosyl residues with a net positive charge that promotes intercellular aggregation and attachment of cells to inert surfaces. The *ica* operon consists of four biosynthesis genes such as *icaA*, *icaD*, *icaB* and *icaC* and a divergently transcribed repressor, *icaR*. Carriage of the *ica* locus is a characteristic of most clinical *S. aureus* strains [62].

One of the reasons the staphylococcal infections are difficult to eradicate is the bacteria's ability to develop community structures known as biofilms by attaching to different surfaces (tissues, catheters and medical devices), and often occur in areas of the body that are not easily accessible for treatment [63].

Biofilm is an assemblage of microbial cells that are irreversibly associated to a surface and embedded in a protective extracellular polymeric matrix. Biofilm-associated organisms have proteins production and genes expression modification compared to their planktonic counterparts [64].

Biofilm formation can be divided into three major stages: initial attachment, development/maturation of the biofilm and dispersion.

During initial attachment, bacteria adhere to the available surface and in case of abiotic one its conditioning is important through various physiochemical parameters: chemical composition of the material, hydrophobicity, electrostatic charges, surface energy and surface roughness and in the case of biotic adhesion: serum and tissue protein adsorption [65].

The final stage of biofilm development is the detachment of cells from the biofilm colony and their dispersal into the environment, which contributes to biological dispersal, bacterial survival and disease transmission. Like other stages of biofilm development, dispersal is a complex process that involves numerous environmental signals, signal transduction pathways and effectors [66].

The biofilm matrix is a complex structure that contains extracellular DNA (eDNA), both from lysed bacteria and potentially from host neutrophil cell death [53], proteinaceous adhesins directly associated with bacteria in the biofilm or free in the biofilm matrix [67], recycled

cytoplasmic proteins that moonlight as components of the extracellular matrix [68], the extracellular polysaccharide intercellular adhesin (PIA), teichoic acids [53]. The matrix can impede the access of certain types of immune defenses, such as macrophages [69].

Cell adhesion and subsequently biofilm formation are processes mediated by covalently and non-covalently cell wall proteins and non-protein factors. For *S. aureus* more than 20 adhesins were identified [53].

Switching between planktonic and biofilm-forming modes represents a major life style change for microbes, and has been shown to be a tightly regulated process [70] through quorum sensing (QS) which is a cell-cell communication mechanism in which bacteria secrete and sense small diffusible molecules called autoinducers (AIs) to coordinate social activities, such as bioluminescence, biofilm formation, swarming behavior, antibiotic production and virulence factor secretion [71].

Staphylococcal biofilm formation is affected by growth conditions (e.g., NaCl, glucose, human plasma, etc.) and is controlled by multiple global regulators such as SarA, Agr, SigB and Sae. The Sae-regulon includes both the factors promoting biofilm formation (i.e., Coa, Emp, Eap, FnBPA, FnBPB, Hla and Hlb) and biofilm dispersal factors (nuclease and proteases) and depending on growth conditions and strain backgrounds, the Sae system could affect biofilm formation either positively or negatively [72].

Bacteria in biofilms can tolerate ten to thousand fold higher levels of antibiotics than the genetically equivalent planktonic bacteria. Staphylococcal biofilms cause biomaterial-associated infections which do not respond to antimicrobial treatment often requiring removal of the same leading to substantial morbidity and mortality. It has also been observed that biofilms harbour persister cells and small colony variants [73], whereas planktonic persisters are eliminated by the immune system *in vivo*, persisters in biofilms serve as a shield evading the immune response and a reservoir of such shielded persisters is a potential source for the emergence of heritable antibiotic resistance [14].

4. *Staphylococcus aureus* soluble virulence factors

S. aureus secretes numerous exotoxins, including polypeptides that destroy the integrity of the host cell plasma membrane. These polypeptides are pore-forming toxins (PFT): α-hemolysin and the bi-component leukocidins γ-hemolysin, β-hemolysin, the Panton Valentine leukocidin (PVL), LukED, and LukGH/AB and phenol soluble modulins (PSMs) [74].

α-Hemolysin is the most characterized virulence factor of *S. aureus*. Upon binding to the cell surface, α-hemolysin forms pores that allow the transport of molecules such as K+ and Ca^{2+} ions, leading to necrotic death of the target cell.

S. aureus possesses several other PFTs in addition to α-hemolysin that for pore formation involves two polypeptides that have been named S (slow) and F (fast) based on their electrophoretic mobility. The PFTs include (i) γ-hemolysin corresponding to two combinations of a

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S component (HlgA or HlgC) with a F component (HlgB); (ii) the PVL made of LukS-PV and LukF-PV; (iii) LukED and (iv) LukGH, also known as LukAB [74]

The γ hemolysin variant and leukocidin E-D gene, as well as other genes encoding exotoxins, were detected evenly in HA and CA-MRSA strains, while sec and sek genes were found only in CA-MRSA strains [75].

β-Hemolysin is a neutral sphingomyelinase, it hydrolyses a plasma membrane lipid—sphingomyelin and does not form pores in the plasma cell. β-hemolysin's enzymatic activity is required for its hemolytic activity [74].

δ-Hemolysin is a small amphipathic α-helix-structured peptide (26 AA). Its hemolytic activity can be realized by forming transmembrane pores, affecting the membrane curvature or acting as detergent to solubilize the membrane [76].

This family of cytotoxic peptides includes new peptides termed PSM. PSMs represent a secreted α-helical peptides produced by different *Staphylococcus* species. PSMs encoding genes are able to activate and lyse human neutrophils and are generated at high concentrations by CA-MRSA strains [77]. The *psm-mec*, was the first PSMs gene found within an SCCmec MGE and was linked to the class A mec gene complex present in SCCmec types II, III and VIII, with a conserved location next to the *mecI* gene [74, 78].

Protein A prevents the opsonization and phagocytosis by ineffectually binding the Fc region of IgG. It also initiates a proinflammatory cascade in the airway by activating tumor necrosis factor receptor 1 (TNFR1) and B cells in concert with other ligands. MRSA strains with certain *spa* types have a decreased ability to invade human cells in vitro, revealing an association with certain *spa* types and virulence [18].

S. aureus produces a group of toxins called the toxic shock syndrome toxin-1 (TSST-1) and enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and SEI) and the exfoliative toxins A and B (involved in staphylococcal scalded skin syndrome). Cytolytic toxins form pores of holes called β-barrel pores in the plasma membrane. This leads to leakage of the cell's content and lysis of the target cell [79].

PVL is a bi-component exotoxin transmitted by bacteriophages, encoded by two genes, *lukF-PV* and *lukSPV*. PVL genes are carried by CA-MRSA strains and in a small proportion by clinical MSSA strains. Therefore PVL plays an important role in fitness, transmissibility and virulence, but the role of PVL in the pathogenesis of CA-MRSA infections is not fully understood [80]. PVL genes are spreading among *S. aureus* strains by clonal expansion and horizontal transfer. There have been demonstrated that β-lactam antibiotics increased the production of PVL in vitro through transcriptional activation [81, 82]. PVL inserts itself into the host's plasma membrane and forms a pore of a hole. PVL exhibits a high affinity toward leukocytes. PVL can inactivate mitochondria and induce apoptosis. In animal models, PVL has been shown to be dermonecrotic [18], perhaps explaining the pathobiology of the characteristic skin lesions associated with CA-MRSA SSTIs. That the presence/absence of PVL genes in MRSA strains did not interfere with strain virulence in mouse models of sepsis and SSTI, and their presence did not decrease neutrophil survival in in vitro assays [18].

α-Toxin lyses immune cells like macrophages and lymphocytes, alters platelet morphology, which increased thrombotic events associated with *S. aureus* sepsis [83]. The nucleases, proteases, lipases, hyaluronidase and collagenase convert local host tissue into nutrients required for bacterial growth.

Arginine catabolic mobile element (ACME) is a large MGE that plays an important role in the growth, transmission and pathogenesis of CA-MRSA. Two main gene clusters identified as *arc* genes (*arcA*, *arcB*, *arcC* and *arcD*) and the *opp* genes (*opp-3A*, *opp-3B*, *opp-3C*, *opp3-D* and *opp3-E*) are recognized to be virulence factors [84].

5. Regulation of virulence factors expression in *Staphylococcus aureus*

In *S. aureus*, virulence factors production is coordinated by different regulators, for example, DNA binding proteins (e.g., SarA and its homologues), two-component signaling systems (e.g., ArlRS, AgrAC, SaeRS and SrrAB) and alternative sigma factor B [72].

Among the regulatory elements, the Agr (the accessory gene regulator) system is the only characterized QS system in *S. aureus* and controls the expression of approximately 150 genes [85]. Agr system regulation depends on cell density. During initial stage of colonization, when there is a low cell density, the Agr QS system is expressed low, but when the biofilm reach maturation and the cell density is high, Agr activity increases and upregulates secreted virulence factors. The *agr* regulation of the proteases is via Rot, whose transcriptional repression of the proteases is relieved when *agr* is induced [65, 86].

S. aureus produces AI-2 through the functional *luxS* gene it has. Due to the dual function of LuxS and the absence of genomic evidence of established AI-2 receptors, the AI-2 quorum-sensing function in *S. aureus* has been intangible, until now [71].

Sigma B (SigB) is an alternative sigma factor of RNA polymerase that is activated in stress response and modifies gene expression. SigB upregulates the expression of different factors involved in initial stages of biofilm formation like coagulase, FnBPA and clumping factor [87]. It also controls negative factors that are associated with a planktonic phenotype and seeding dispersal, including enterotoxin B, cysteine protease (SplB), serine protease (SplA), the metalloprotease Aur, staphopain, leukotoxin D and β-hemolysin [53].

The *sar* (Staphylococcal accessory regulator) locus produces three transcripts from three separate promoters, all of which contain the ORF for the DNA-binding protein SarA [53]. SarA also directly regulates several genes that affect biofilm formation. There have been demonstrated that the mutation of *sarA* gene in the USA300 clone limits accumulation of α-toxin and PSMs through the increased production of extracellular proteases rather than from transcription of the *hla* or *agr* genes [88].

6. Conclusions

Staphylococcus spp. are common residents of the normal human and animal microbiota, but in certain favoring conditions, they can surpass the anti-infectious defense barriers and become

opportunistic pathogens. Staphylococci can cause numerous types of infections some of them potentially fatal due to their numerous virulence factors, biofilm formation capacity and antibiotic resistance mechanisms. Taking into account the increased incidence and spread of MRSA and multiple-drug resistant strains, a better knowledge of the virulence and pathogenicity mechanisms and of their relationships with resistance, as well as of the quorum sensing mechanisms is essential for the development of novel anti-staphylococcal strategies, targeting the expression of virulence factors or of their regulatory mechanisms.

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Conflict of interest

There is no conflict of interest.

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Chapter 6

Antimicrobial Susceptibility Pattern of *Staphylococcus aureus*

Tebelay Dilnessa

Additional information is available at the end of the chapter

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Abstract

Staphylococcus aureus particularly methicillin-resistant *Staphylococcus aureus* (MRSA) strains is one of the major causes of community and hospital-acquired bacterial infections. They are also becoming increasingly multidrug resistant and recently developed resistance to vancomycin, which has been used successfully to treat MRSA for many years. In vitro determination of drug resistance patterns of *S. aureus* is critical for the selection of effective drugs for the treatment of staphylococci infections. The main aim of this review was to determine the prevalence of drug-resistant *S. aureus* strains from different clinical specimens throughout the world. Various types of research study designs such as cross-sectional and retrospective and laboratory techniques like Kirby Bauer, agar dilution, and E tests were used. The result of each study was narrated accordingly.

Keywords: prevalence, MRSA, beta-lactamase, antimicrobial susceptibility pattern

1. Introduction

Staphylococcus aureus particularly methicillin-resistant *Staphylococcus aureus* (MRSA) strains is one of the major causes of community and hospital-acquired bacterial infections. They are also becoming increasingly multidrug resistant and recently developed resistance to vancomycin, which has been used successfully to treat MRSA for many years. In vitro determination of drug resistance patterns of *S. aureus* is critical for the selection of effective drugs for the treatment of staphylococci infections. The main aim of this review was to determine the prevalence of drug-resistant *S. aureus* strains from different clinical specimens throughout the world. Different types of research study designs such as cross-sectional and retrospective and laboratory techniques like Kirby Bauer, agar dilution, and E tests were used. The result

of each study was narrated with respect to antimicrobial susceptibility pattern of *S. aureus* to various drugs accordingly.

A study on methicillin resistance against *S. aureus* in Trinidad and Tobago was conducted by Akpaka et al. [1]. Of 1912 *S. aureus* isolates recovered from different clinical samples, 12.8% were found out to be methicillin (oxacillin) resistant. The highest (86%) of the isolates were obtained from wound swabs and the least from urine (0.4%) specimens. About 85% of methicillin-susceptible *S. aureus* (MSSA) were sensitive to commonly used antimicrobials in the country. On the other hand, all MRSA isolates were resistant to ceftriaxone, erythromycin, gentamycin, and penicillin but were 100% sensitive to vancomycin, rifampin, and chloramphenicol.

Similar study was carried out by Orrett and Land [2] in Trinidad and Tobago. In this study, 2430 isolates of *S. aureus* strains recovered from various clinical sources, from hospital and community practices, were analyzed. The prevalence of MRSA varied with the type of clinical sample. The prevalence of MRSA from surgical/burn wound was the highest (60.1%) followed by urine (15.5%) and pus/abscess (6.6%), respectively. The prevalence of MSSA also varied with the type of clinical samples. The major sources of MSSA were surgical/burn wounds, pus/abscess, and upper respiratory tract specimens with rates of 32.9, 17.1, and 14.3%, respectively. Furthermore, 109 (4.5%) *S. aureus* strains were isolated from sputum, 201(8.3%) from blood, and 95(4%) from eye infection. Clinical specimens each accounting less than 3% of the total include the vagina, ear, and CNS. With regard to the antimicrobial susceptibility profile of the isolates, the greatest prevalence of resistance of MRSA was seen for erythromycin (86.7%) and clindamycin (75.3%). Resistance rates among MSSA were highest for ampicillin (70%).

Oxacillin-resistant and multidrug-resistant *Staphylococcus aureus* in Lima, Peru, was studied by Seas et al. [3]. *S. aureus* isolates were recovered from the blood, sterile body fluids (e.g., cerebrospinal fluid, peritoneal, joint, and pericardial fluids), urine, skin and soft tissue, lungs, abscesses, surgical wound sites, and catheters. Of 103 strains isolated, 70 (68%) were MRSA. In the United States, the prevalence of MRSA in skin and soft tissue infections was conducted by Frazee et al. [4]. Among 137 study subjects, 119 *S. aureus* isolates were recovered of which MRSA was present in 51% of infection site cultures. Of 119 isolates 89 (75%) were MRSA. All MRSA strains were susceptible to trimethoprim/sulfamethoxazole, 94% to clindamycin, 86% to tetracycline, and 57% to levofloxacin. Similarly, results of this study revealed that the prevalence of MRSA was 59%. Moran et al. [5] conducted MRSA prevalence study in patients with skin and soft tissue infections. In this study a total of 422 patients with skin and soft tissue infections were enrolled. *S. aureus* was isolated from skin and soft tissue infection in 320 (76%) patients of which 249 (78%) of the *S. aureus* isolates were MRSA. This study revealed that the isolation rate of MRSA varies with respect to clinical sample. MRSA isolated from abscesses, purulent wounds, and cellulitis with purulent exudates accounted 61, 53, and 47%, respectively.

The prevalence of MRSA across the European countries from 1999 to 2002 was analyzed by Tiemersma et al. [6]. In this study a total of 50,759 *S. aureus* isolates were collected from 495 hospitals in 26 countries. The prevalence of MRSA varied from 1% in Northern Europe to 40% in Southern and Western Europe. The study also has shown that the prevalence of MRSA

increased significantly in countries such as Belgium, Germany, Ireland, the Netherlands, and the United Kingdom, while the prevalence of MRSA showed a decrease in Slovenia. In addition this study revealed that MRSA was more frequently isolated from men than women and patients with blood culture positive for MRSA were older than patients with MSSA.

Many studies on the prevalence of MRSA have been conducted in India. A total of 1426 wound swabs were taken from 450 high-risk patients by Vidhani et al. [7] of which *S. aureus* was isolated from 188 patients (41.8%) and out of which 97 (51.6%) patients were found to be MRSA. A marked difference in antibiotic sensitivity pattern of MRSA and MSSA isolates was reported. According to the results of this study, none of the MRSA isolate was found to be sensitive to penicillin and amoxicillin. However, 6 (5.5%) and 12 (11%) MSSA were sensitive to penicillin and amoxicillin. A total of 85 (77.9%) of MSSA were sensitive to cefotaxime, while only 17 (21.5%) of MRSA were sensitive to this antibiotic. Sensitivity to macrolide group of antibiotics like erythromycin and roxithromycin was seen in 77 (70.6%) of MSSA in comparison to 14 (17.7%) of MRSA. Susceptibility test results of this study further showed that among the aminoglycosides maximum sensitivity of MSSA was seen with amikacin 74 (67.9%), while only 21 (26.6%) of MRSA were sensitive to the same antibiotic. A total of 53 (67%) of MRSA and 76 (69.7%) of MSSA were found to be sensitive to fluoroquinolone group, that is, ofloxacin. All *S. aureus* isolates (MRSA and MSSA) were found to be uniformly sensitive to vancomycin which is the drug of choice for treating infections caused by MRSA.

Another study conducted by Rajendra Goud et al. [8] revealed a prevalence 29.76% of community-associated MRSA. All community-associated MRSA were resistant to methicillin and penicillin, while resistance to erythromycin and vancomycin was 65 and 1.12%, respectively, but all MRSA isolates were sensitive to linezolid. A third study conducted by Sharma and Mall [9] found out that out of 200 nasal samples, *S. aureus* was recovered from 97 patients, and of these, 23 isolates were MRSA. The drug resistance patterns of MRSA isolated from clinical specimens, and carrier screening samples were found to be highly variable. Almost all the MRSA strains (91.3%) screened from nasal samples were resistant to amikacin, 86.95% to kanamycin and cloxacillin, 78.26% to ciprofloxacin, 56.52% to erythromycin, 52.17% to chloramphenicol, and 34.78% to both tetracycline and gentamycin. The production of β-lactamase enzyme in MRSA was found to be 19 (82.6%). Chandrashekhar et al. [10] isolated 312 *S. aureus* strains of which 177 (56.75%) were found to be MRSA. Susceptibility profile of this study showed that all MRSA were resistant to penicillin, followed by erythromycin (91.5%), ampicillin+ sulbactam (90.4%), amoxicillin (83.6%), norfloxacin (81.4%), cefuroxime (78.5%), and amikacin (25.4%). However, no strains were resistant to vancomycin. Similar study carried out by Kaur et al. [11] revealed that 27 out of 70 (38.6%) *S. aureus* isolates were MRSA.

A number of similar studies were carried out in other Asian countries. A study carried out in Tehran by Vahdani et al. [12] exhibited marked variation in the drug susceptibility of MRSA. The results of this study showed that all the 90 MRSA isolates were resistant to penicillin (100%), ampicillin (92%), and cefotaxime (93%). Vancomycin and chloramphenicol were the most effective antibiotics, and only 7 and 14% of isolates were resistant, respectively. Nitrofurantoin, gentamycin, amikacin, ciprofloxacin, and other cephalosporins like cefepime and cefazolin were better active than penicillin, ampicillin, and cefotaxime. This study showed that 44% of hospital-acquired MRSA strains were resistant to co-trimoxazole. Akhter et al. [13]

in Karachi isolated MRSA and determined the drug susceptibility of pattern of both MRSA and MSSA. A total of 87 strains of *S. aureus* were recovered from various clinical samples by the authors. Of these, 66 (75.8%) strains were recovered from various swabs and 21 (24.13%) from blood. Of the isolates 20 (22.9%) were methicillin resistant. In this group high resistance was found to cloxacillin (100%), co-trimoxazole (95%), erythromycin (70%), and gentamicin (55%), and low resistance was observed to ciprofloxacin (30%). In MSSA 0% resistance was seen to ciprofloxacin and chloromycetin, and high resistance was found to co-trimoxazole (98.5%) and penicillin (73.13%). Both MRSA and MSSA were 100% sensitive to vancomycin. A total of 139 MRSA were isolated by Kaleem et al. [14] in Pakistan. Of this most of the MRSA were isolated from pus samples. As far as their drug susceptibility is considered, all of the isolated MRSA were found to be susceptible to vancomycin and linezolid. Furthermore, 130 isolates (94%) were susceptible to teicoplanin and minocycline, whereas 93% of isolates were sensitive to chloramphenicol and 91% were sensitive to tetracycline. Only 38 and 22% of the isolates were susceptible to fluoroquinolones and macrolides, respectively.

A good number of research work on the prevalence, rate of isolation, and drug susceptibility profile of MRSA have been carried out in Africa. A study carried out by Ojulong et al. [15] investigated 188 pus swabs collected from patients with surgical site infections. Out of 54 (28.7%) *S. aureus* isolates, 17 (31.5%) were found out to be MRSA. Resistance rates of MRSA were found out to be 88.2% for trimethoprim-sulfamethoxazole, 88.2% for erythromycin, 58.8% for gentamycin, 70.6% for ciprofloxacin, and 88.2% for chloramphenicol, and all MRSA isolates were found to be sensitive to vancomycin and clindamycin. A study carried out in Sudan by Alamin et al. [16] recovered 85 *S. aureus* strains of which 21 (24%) were isolated from nasal cavity, 26 (31%) from skin surface, 22 (26%) from wounds, and 16 (19%) from the throat. Out of 85 isolates, 25 were found out to be MRSA.

Okwu et al. [17] in Nigeria examined 120 samples taken from the nose. Of these 22 (18.3%) were found to be positive for *S. aureus*, and 13 (10.8%) of the isolates were oxacillin resistant. Their studies also depicted that seven (11.7%) MRSA strains were obtained from females, while six (10%) strains were from males. Also, 12 (19.4%) *S. aureus* and 7 (11.3%) MRSA were isolated from the age group of 9–14 years, while 10 (17.3%) isolated of which 6 (10.3%) were MRSA isolated the age groups of 3–8 years. Furthermore, the isolates were resistant to ampicillin (100%), cloxacillin (100%), penicillin (100%), tetracycline (82%), chloramphenicol (73%), erythromycin (68%), gentamicin (64%), streptomycin (56%), and oxacillin (55%). Another study conducted by Olowe et al. [18] in the same country, Nigeria, depicted that out of 67 *S. aureus* isolates, 32(47.8%) were resistant to methicillin. High prevalence of MRSA, 13 (19.4%), was isolated from wound, while urine sample had the least, 1(1.5%). High resistance levels (87.5%) were detected against penicillin and tetracycline, while gentamicin and vancomycin recorded the least resistance levels of 62.5 and 6.3%, respectively. The starch paper analysis confirmed the presence of beta-lactamase production in all the isolates tested (100%). Similar study was conducted to detect beta-lactamase production in the same country by Efuntoye et al. [19]; of the 95 isolates tested. A total of 79 (83.2%) were beta-lactamase-producing strains.

In Ethiopia, a retrospective study on the prevalence of MRSA was conducted by Geyid et al. [20]. The results of this study showed that among 249 *S. aureus* isolates 75 (30.5%) were found

out to be MRSA, while 173 (69.5%) were MSSA. With regard to antibiotic susceptibility pattern of the isolates, vancomycin and clindamycin were effective against all *S. aureus* isolates. The presence of beta-lactamase production was determined in the 355 *S. aureus* isolates, and 252 (71%) were found to be beta-lactamase producers. Furthermore, 47 (62%) of the MRSA isolates and 140 (81%) of the MSSA isolates were beta-lactamase-positive strains. The sensitivity pattern of all the *S. aureus* isolates against 11 common drugs indicated that the majority (80%) of the MRSA strains were multidrug resistant, while 4 (8%) were not resistant to any of the drugs tested. A total of 41 (54%) MRSA strains were both beta-lactamase producers and multidrug-resistant isolates. Another study carried out in Felege Hiwot Referral Hospital, Bahir Dar, showed that 55% of *S. aureus* isolates were MRSA [21].

Similarly, in a study conducted by Dilnessa et al. [22], of 1360 clinical specimens analyzed, *S. aureus* was recovered from 194 (14.3%). Rate of isolation of *S. aureus* with regard to clinical specimens was the highest in pus 118 (55.4%). No *S. aureus* was isolated from CSF and urethral discharge. Out of 194 *S. aureus* isolates, 34 (17.5%) were found out to be MRSA and the remaining 160 (82.5%) were MSSA. A total of 98 (50.5%) *S. aureus* isolates were multidrug resistant, and the highest isolates were resistant to penicillin 187 (96.4%) and least resistant for clindamycin 23 (11.9%) and vancomycin 10 (5.1%). MRSA strains were 100% resistant to penicillin G, erythromycin, and trimethoprim-sulfamethoxazole and least resistant to vancomycin 10 (29.4%). Out of 194 *S. aureus* isolates, 153 (79.0%) were beta-lactamase producers (**Table 1**).

Factors that could contribute to variations in the prevalence rate of MRSA and vancomycin could be due to differences in the length of study period, number of study sites, sample size,

Authors (publication year)	Country	Sample size	No. of *S. aureus* (N/%)	MRSA (N/%)	MSSA (N/%)	VRSA (%)	MDRSA (%)
Moran et al. (2006)	USA	422	320 (76)	249 (78.0)	71 (22.0)	—	—
Ojulong et al. (2009)	Uganda	188	54 (28.7)	17 (31.5)	37 (68.5)	0	—
Sharma and Mall (2011)	India	200	97 (48.5)	23 (23.7)	74 (76.3)	—	—
Okwu et al. (2012)	Nigeria	120	22 (18.3)	13 (59.1)	9 (40.9)	—	100
Akpaka et al. (2006)	Spain	—	1912	244 (12.8)	1668 (87.2)	0	—
Geyid et al. (1991)	Ethiopia	17,142	249 (1.4)	76 (30.5)	173 (69.5)	0	80
Dilnessa et al. (2016)	Ethiopia	1360	194 (14.3)	34 (17.5)	160 (82.5)	5.1	50.5
Olowe et al. (2012)	Nigeria	—	67	32 (47.8)	35 (52.2)	6.3	100
Vidhani et al. (2000)	India	450	188 (41.7)	97 (51.6)	91 (48.4)	—	79.5
Alamin et al. (2013)	Malaysia	—	85	25 (29.4)	60 (70.6)	8	—

MRSA, Methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; MDRSA, multidrug-resistant *S. aureus*; VRSA, vancomycin-resistant *S. aureus*

Table 1. Comparison of different literatures with respect to methicillin- and vancomycin-resistant pattern of *Staphylococcus aureus* in different countries.

and sample type, and the lab procedures employed can be mentioned. The isolates were multidrug resistant to several combinations of the tested antibiotics. According to Magiorakos et al. [23], MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories. Over all drugs such as gentamicin, amoxicillin-clavulanate, clindamycin, cefuroxime, vancomycin, and cephalothin had relatively lower resistance.

2. Conclusion

The prevalence of *S. aureus* and MRSA varies appreciably based on the type of clinical samples. Pus is the main source of *S. aureus* and MRSA than other samples in hospital settings. The prevalence of MRSA stains obtained from different studies varies based on geographical location. Many MRSA strains were multidrug resistant, and a good number of the isolates were also resistant to vancomycin, the drug of choice for treating multidrug-resistant MRSA infections. Reducing this burden by good infection control practices such as strict hand washing, by identifying MRSA carriers, and treating them, the prudent use of antimicrobial agents is recommended. Beta-lactamase production plays a great role for acquisition of MRSA. Physicians should prescribe drugs after the sensitivity pattern of the microbe is known. Additionally, large-scale longitudinal study is needed to determine CA-MRSA and HA-MRSA. Further phenotypic and genotypic studies are needed to establish and clarify the genetic mechanism behind susceptibilities to antibiotics.

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Chapter 7

Resistance of Staphylococci to Macrolides-Lincosamides-Streptogramins B (MLS_B): Epidemiology and Mechanisms of Resistance

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Additional information is available at the end of the chapter

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Abstract

A total of 92 genes that confer resistance to MLS antibiotics have been described to date. They can be roughly divided into three groups, depending on the mechanisms by which they confer resistance to one or all of these groups of antibiotics. Three main mechanisms of resistance to MLS antibiotics have been described: methylation of rRNA (target modification), active efflux and inactivation of the antibiotic. Target modification is achieved through the action of the protein product of one of more than 42 different *erm* (erythromycin rRNA methylase) genes. They confer cross resistance between macrolides, lincosamides and streptogramin B (so-called MLS_B resistance) and evoke most concerns. Active efflux and inactivating enzymes (M and L) represent two additional mechanisms of resistance that are targeted only to particular antibiotics or antibiotic classes. Based on the mechanisms of resistance, various resistant phenotypes are expressed. The most prevalent phenotypes are MLS_B (constitutive or inducible), which is associated with the presence mainly of *ermA* and *ermC* genes, followed by the MS_B phenotype due to the presence of *msrA* gene. In livestock *S. aureus* strains, such as CC 398, other genes such as *ermT*, *lnuA*, *lsaE* and *mphC* genes are detected.

Keywords: staphylococci, MLS_B, resistance, genes

1. Introduction

Resistance to macrolides-lincosamides and streptogramins B (MLS_B antibiotics) is associated with three main mechanisms: (1) methylation of rRNA (target modification), (2) active efflux and (3) enzymatic inactivation. Till date, a total of 92 genes, conferring resistance to MLS_B antibiotics, have been described. The most common genes are *erm*, which encode rRNA

methylases, resulting in the target modification of these antimicrobial agents. More than 42 different *erm* genes have been described to date; bacteria, that carry *erm* genes, express cross-resistance to all these classes of antimicrobial agents. On contrary, genes encoding pumps for active efflux (*msrA* and *lsa*) or enzymes for drug inactivation (*lnu* and *mphC*) confer resistance only to particular antibiotics. Based on the mechanisms of resistance, various resistant phenotypes are expressed. The most prevalent phenotypes are MLS_B (constitutive or inducible), which, in staphylococci, are associated with the presence mainly of *ermA* and *ermC* genes, followed by the MS_B phenotype due to the presence of *msrA* gene. In livestock *S. aureus* strains, such as CC 398, other genes such as *ermT*, *lnuA, lsaE* and *mphC* genes are detected [1–4].

The macrolide group of antibiotics includes natural members, prodrugs and semisynthetic derivatives. The chemical structure of macrolides is characterized by a large lactone ring containing from 12 to 16 atoms to which are attached, via glycosidic bonds, one or more sugars. Erythromycin, whose lactone ring contains 14 atoms, is the oldest molecule (1952), whereas all second-generation macrolides, like roxithromycin and clarithromycin, are hemisynthetic derivatives of erythromycin. Azithromycin is the only macrolide with 15 carbon atoms. Azithromycin, which is produced through the introduction of a nitrogen atom into the macrolide nucleus at C10, exhibits (1) improved penetration into macrophages, fibroblasts and polymorpho-neutrophils, (2) increased accumulation within acidified vacuoles and (3) extended half-life. Additionally, azithromycin shows improved activity against Gram-negative bacteria and other pathogens associated with parasitic infections. Spiramycin and josamycin are macrolides with 16 carbon atoms. All chemical modifications of macrolides were made in order that their properties and action are optimized.

Although the structure of lincosamides is different from the structure of macrolides, they present a similar action spectrum. Lincomycin, which was isolated in 1962, is a fermentation product of *Streptococcus lincolnensis*. Clindamycin (7-chloro-7-deoxy-lincomycin) is a semisynthetic derivative of lincomycin, produced by substitution of the C7 bearing a hydroxyl group with a chlorine atom. Clindamycin exhibits higher antibiotic activity and digestive absorption.

Type-A streptogramin includes cyclic-poly-unsaturated macrolactones: virginiamycin M, pristinamycin IIA and dalfopristin. Type-B streptogramin consists of the cyclic hexadepsipeptide compounds virginiamycin S, pristinamycin IA and quinupristin. Until now, only three streptogramins have been marketed either for treatment or growth promotion: virginiamycin, pristinamycin and quinupristin-dalfopristin. Virginiamycin, a mixture of virginiamycin M (type A streptogramin) and virginiamycin S (type B streptogramin), has been used mainly as growth promoter feed additive in commercial animal farming in the United States and Europe. In contrast, pristinamycin has been used orally and topically in human medicine only in France. Qiunupristin-dalfopristin, in a 30:70 mixture (Synercid), was approved in 1999 for the treatment of serious infections caused by multidrug resistant Gram-positive pathogens, including vancomycin-resistant *Enterococcus faecium* and methicillin-resistant staphylococci (MRS).

MLS_B antibiotics share a similar mode of action because they inhibit protein synthesis by targeting the peptidyl transferase center within the 50S subunit (23 s rRNA) of the bacterial ribosome [5]. We note that the bacterial ribosomes are 70S particles comprising of two subunits, 30s and 50S, which are made of RNAs enveloped by proteins; 50S is composed of 5S, 23S rRNAs and 36 proteins (L1-L36) [6, 7].

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Although the peptidyl transferase center is the main target site for many antibiotics, the exact mechanism for its activity is still unclear [8]. Overall, the inhibitory action of antibiotics is not only determined by their interaction with specific nucleotides. MLS_B could also inhibit peptidyl transferase by interfering with the proper positioning and movement of the tRNAs at the peptidyl transferase cavity [9, 10].

2. Antibacterial spectrum of MLS_B

The spectrum of MLS_B includes mainly Gram-positive microorganisms (streptococci, staphylococci); however, some of them also have activity against Gram-negative microorganisms (*Bordetella pertussis*, *Campylobacter*, *Helicobacter*, *Legionella*, *Moraxella catarrhalis*), anaerobes, intracellular pathogens (*Chlamydia* and *Rickettsia*) and *Mycobacterium avium* [11, 12].

It is known that some Gram-positive species have intrinsic resistance to some of them. *Enterococcus faecalis*, *E. avium*, *E. gallinarum* and *E. casseliflavus* express resistance to lincosamides. Among staphylococci, *S. cohnii, S. xylosus* and *S. sciuri* are also resistant to lincosamides [11, 12].

3. Mechanisms of acquisition of resistance to MLS_B

Staphylococci resist MLS_B antibiotics in three ways: (1) through target-site modification by methylation or mutation that prevents the binding of the antibiotic to its ribosomal target, (2) through efflux of the antibiotic and (3) by drug inactivation. Modification of the ribosomal target confers broad-spectrum resistance to macrolides, lincosamides and streptogramin B, whereas efflux and inactivation affect only some of these molecules [12].

3.1. Ribosomal methylation

The most widespread mechanism of resistance to MLS_B in Gram-positive bacteria, including both *Staphylococcus aureus* and coagulase-negative staphylococci (CNS), is the methylation of ribosomes, which is the target of MLS antibiotics. Methylation of ribosomes leads to resistance to macrolides, lincosamides and streptogramins B (MLS_B phenotype) [13]. The MLS_B phenotype is conferred by erythromycin ribosome methylases (Erm), which are encoded by *erm* genes. *erm* genes have been reported in a large number of microorganisms [14].

Erm proteins, encoded by *erm* genes, dimethylate the A2058 residue of 23S rRNA [13], which is located within the conserved domain V of 23S rRNA in the bacterial ribosome. Domain V of the 23S rRNA plays a key role in the binding of MLS_B antibiotics. Methylation of 23S rRNA impairs binding of macrolides, lincosamides and streptogramins B, which accounts for the cross-resistance to these drugs. A wide range of microorganisms, including Gram-positive bacteria, spirochetes and anaerobes, which are targeted for MLS_B antibiotics, express Erm methylases.

More than 42 *erm* genes have been reported so far [14]. In bacteria, *erm* genes are usually carried by plasmids and transposons that are able to move independently. Four major classes are detected in microorganisms: *ermA*, *ermB*, *ermC* and *ermF* [13, 14]. *ermA* and *ermC* typically are staphylococcal gene classes.

3.2. Antibiotic efflux

In Gram-positive organisms, acquisition of macrolide resistance by active efflux is caused by two classes of pumps, members of the ATP-binding-cassette (ABC) transporter superfamily and of the major facilitator superfamily (MFS). ABC transporters require ATP to function and are usually formed by a channel comprising two membrane-spanning domains and two ATP-binding domains located at the cytosolic surface of the membrane [12].

The first determinant encoding ABC transporter in staphylococci was the plasmid-borne *msr*(A) gene [15]. The *msr*(A) gene encodes an ABC transporter protein with two ATP-binding domains. The nature of the transmembrane component of the MsrA pump remains unknown. In nature, a fully operational efflux pump is a multicomponent system that is composed of proteins encoded by *msr*(A) and chromosomal genes. MsrA pump has specificity for 14- and 15-membered macrolides and type B streptogramins (the MS_B phenotype) [15]. MS_B resistance phenotype is inducibly expressed by 14- and 15-membered macrolides, whereas streptogramins B are not inducers. *msrA*-positive strains are fully susceptible to clindamycin, since this antibiotic is neither an inducer nor a substrate for the pump.

However, latter, the combined resistance to lincosamides, pleuromutilins and streptogramin A (S_A), referred as the PLS_A phenotype, was found to be associated with the presence of the ARE subfamily of class 2 ATP-binding cassette (ABC) ATPases, a class of ABC proteins made up of two homologous ABC ATPase domains separated by a flexible linker without any identifiable transmembrane domains [16–18]. The flexible linker between each ATPase domain is presumed to be the drug-binding region of the ARE proteins. The *vga*-, *lsa*- and *sal*-like genes, encoding ABC transporters of the Vga, Lsa, or Sal families confer the PLS_A resistance phenotype. These genes have been mainly identified in staphylococci causing food-borne diseases [19–26].

3.3. Enzymatic inactivation

Enzymatic inactivation confers resistance to structurally related antibiotics only. Esterases and phosphotransferases, encoded by *ere* and *mphC* genes, respectively, confer resistance to erythromycin and other 14- and 15-membered macrolides but not to lincosamides [27–30] .

In addition, lincosamide nucleotidyl transferases encoded by *lnu*(A) (formerly *linA*) and *lnu*(B) (formerly *linB*) genes in staphylococci (*S. aureus* and coagulase-negative staphylococci) inactivate lincosamides only [14, 31–33]. In addition, enzymes such as virginiamycin B hydrolase and streptogramin B lactonase, encoded by *vgbA* and *vgbB* genes, which hydrolyze streptogramin B, are rarely found in staphylococci [14, 34, 35].

3.4. Uncommon mechanisms of resistance

Ribosomal mutations (A2058G/U or A2059G) of 23S rRNA gene such as mutations in the *rplV* gene, encoding the L22 ribosomal protein have been reported by Prunier et al. [36]. These rare *Staphylococcus aureus* isolates, recovered from patients with cystic fibrosis after long-term treatment with azithromycin, were cross-resistant to azithromycin and erythromycin.

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On the other hand, *Staphylococcus epidermidis* isolates, which carried the T2504A mutation of 23S rRNA gene were found to be fully resistant to lincomycin, clindamycin, linezolid and pleuromutilins [37].

4. Resistant phenotypes: expression, detection and interpretation

Depending on the mechanism of resistance and on the carriage of respective genes, staphylococci can express various MLS_B resistant phenotypes. Briefly, these types are described as follows.

4.1. MLS_B phenotype (*erm* genotype)

MLS_B phenotype can be expressed as constitutive or inducible [12]. Isolates with a constitutive MLS_B phenotype express high level cross-resistance to macrolides, lincosamides and streptogramin B. In fact, clinical methicillin-resistant strains that are constitutively resistant to MLS_B antibiotics are widespread.

On the other hand, isolates with an inducible MLS_B phenotype express phenotypically only resistance to macrolides and susceptibility to lincosamides. This phenomenon is explained by the fact that, in constitutive resistance, bacteria produce an active mRNA encoding methylase, whereas in inducible resistance, bacteria produce an inactive mRNA, which is unable to encode ribosome methylases. However, in the presence of a macrolide, which acts like an inducer, the mRNA becomes active [38]. The presence of an inducer leads to rearrangements of mRNA, which allow ribosomes to translate the methylase coding sequence.

Inducible expression of *ermA* or *ermC* genes is characterized by dissociated resistance to MLS_B antibiotics. Dissociated resistance to MLS_B antibiotics is due to the differences in the inducing capacity of the antibiotics. For example, 14- and 15-membered ring macrolides, which are inducers, are inactive. Thus, *ermA*- or *ermC*-positive strains are phenotypically resistant to these antibiotics. However, strains remain susceptible to 16-membered ring macrolides, lincosamides, and streptogramins B that are not inducers.

The use of antibiotics being noninducers (such as clindamycin) for treatment of an infection due to a *Staphylococcus aureus* that is inducibly resistant to MLS_B antibiotics is not devoid of risk. In the presence of these antibiotics, constitutive mutants can be selected *in vitro* at frequencies of $\sim 10^{-7}$ cfu. Previous reports have demonstrated the risk of selection of constitutive mutants during the course of clindamycin therapy administered to patients with severe infections due to inducibly erythromycin-resistant *S. aureus* [39, 40]. In addition, the risk for selection of a constitutive mutant is higher if, at the site of infection, staphylococcal inoculum is higher.

According to the rules of EUCAST, if a staphylococcal isolate with an inducible MLS_B phenotype is detected, it must be reported as resistant and considered adding this comment to the report "Clindamycin may still be used for short-term therapy of less serious skin and soft tissue infections as constitutive resistance is unlikely to develop during such therapy."

The *ermA* and *ermC* are the most common determinants in staphylococci [41]. The *ermA* genes are mostly spread in methicillin-resistant strains and are borne by transposons related to Tn*554*, whereas *ermC* genes are mostly responsible for erythromycin resistance in methicillin-susceptible strains and are borne by plasmids. Recently, the *ermT* gene was found to be present in livestock staphylococci [21].

4.2. MS_B-phenotype (*msrA* genotype)

MS_B phenotype is associated with resistance only to 14- (clarithromycin, erythromycin, roxithromycin) and 15-membered ring macrolides (azithromycin) and streptogramin B, while 16-membered ring macrolides (josamycin and spiramycin) and lincosamides remain active [12, 15]. The *msrA* resistance determinant was originally detected in *Staphylococcus epidermidis*, and, since then, it has been found in a variety of staphylococcal species, including *S. aureus*. The MS_B resistance phenotype is inducibly expressed by 14- and 15-membered macrolides. Streptogramins B are not inducers and, therefore, the *msrA*-positive strains are resistant to streptogramins B only after induction. The 16-membered ring macrolides and lincosamides are neither inducers nor substrates for the pump. Thus, *msrA*-positive strains are fully susceptible to these antimicrobials.

Another gene, *msrB* from *Staphylococcus xylosus*, which is nearly identical to the 3′ end of *msrA*, has been reclassified as *msrA* [14]. It contains a single ATP-binding domain but also confers an MS_B phenotype.

Isolates with this phenotype have probably decreased susceptibility to the combination of quinupristin-dalfopristin. Additional tests (see below) are required for its detection.

4.3. M-phenotype (*mphC* genotype)

M-phenotype is associated with the presence of enzymes which inactivate enzymatically only macrolides. Clinical isolates of erythromycin-resistant *S. aureus* and coagulase-negative staphylococci produce phosphotransferases encoded by *mphC* genes [29, 30]. This phenotype must be differentiated from MLS_B-inducible phenotype and from MS_B phenotype. Additional tests (see below) are required for its detection.

4.4. PLS_A-phenotype

PLS_A-phenotype is associated with resistance to lincosamides, pleuromutilins and streptogramins A, while macrolides and streptogramin B remain active [42] . Various genes such as *vgaA*, *vgaC*, *vgaE*, and *lsaE* have been detected in methicillin-resistant *Staphylococcus aureus* (MRSA) of clonal complex (CC) 398 of swine, cattle and poultry origin and shown to confer this resistance phenotype [43, 44].

4.5. L-phenotype (*lnuB* genotype)

L-phenotype is associated with resistance to lincomycin due to the presence of lincosamide nucleotidyl transferases encoded by *lnuA* and *lnuB* genes. Both *lnu*-like genes confer resistance

to lincomycin. Generally, expression of lincosamide nucleotidyl transferases causes increase of lincomycin MICs by only 1 or 2 dilutions [45]. However, *lnu*-like genes do not confer resistance to clindamycin. Indeed, the bactericidal activity of clindamycin, which is already weak against susceptible strains, is totally abolished [45], but the impact of this alteration on the therapeutic efficacy of clindamycin is unknown. Because of dissociated resistance among lincosamides, the detection of L-phenotype is possible only if lincomycin is used, instead of clindamycin.

Although more than 90 genes conferring resistance to macrolides and lincosamides have been described till date, their presence has not turned out to be a successful story for Gram-positive bacteria. This observation, which is in contrast with the success of emergence of *bla* genes in Gram-negative bacteria, could be explained by: (1) a low-level resistance conferred by these genes or (2) a failure of detection.

4.6. S_B-phenotype

S_B-phenotype is expressed by resistance to streptogramin B due to the presence of *vgb*A/B encoding lyases that inactivate the drug. It is very difficult to detect this phenotype since quinupristin is not used alone but combined with dalfopristin. The isolates might express a decreased susceptibility to the combination of quinupristin-dalfopristin .

5. Confirmation methods of resistant phenotypes

Among the different types of resistant phenotypes, the most common are MLS_B (constitutive or inducible), MS_B and M-phenotypes. The clinical microbiology laboratory detects easily and reliably the MLS_B constitutive phenotype: the isolates are fully resistant to macrolides and lincosamides. However, isolates with MLS_B inducible, MS_B and M-phenotypes share the same profile: resistance to macrolides and susceptibility to lincosamides. Therefore, additional test, the double disk diffusion test (D test) is required to be applied.

For the detection of MLS_B inducible resistance, it is recommended to place the erythromycin and clindamycin disks 12–20 mm apart (edge to edge, D test). In disk-diffusion tests, a D-shaped zone, caused by induction of methylase production by erythromycin, can be observed (**Figure 1**). Nowadays, the automated system Vitek II (BoMerieux) has the possibility to detect it.

However, after a negative D test, the differentiation between MS_B and M-phenotypes is more complicated and could be based on the MIC values of erythromycin. Isolates with M-phenotype have often lower MIC values to erythromycin, due to the weak activity of hydrolytic enzymes, than isolates with MS_B-phenotype, which express fully resistance to macrolides. In addition, MS_B-phenotype affects the susceptibility to quinupristin-dalfopristin, decreasing it slowly.

Finally, it is difficult to discriminate isolates with PLS_A-phenotype from those with L-phenotype; both share the same profile, including resistance to lincomycin and susceptibility to erythromycin.

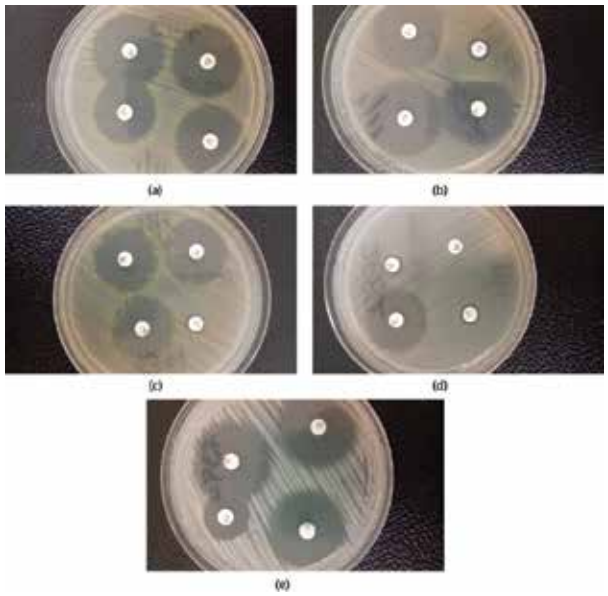

Figure 1. Expression of various resistant-phenotypes: (a) sensitive; (b) MLS_B-inducible phenotype; (c) MS_B-phenotype; (d) L-phenotype and (e) M-phenotype. ERY: erythromycin; CLIN: clindamycin; LIN: lincomycin.

Gene	Primers sequence (5′–3′)	PCR fragment size (bp)
*erm*A	F: TCTAAAAAGCATGTAAAAGAA	645
	R: CTTCGATAGTTTATTAATATTAG	
*erm*B	F: GAAAAGTACTCAACCAAATA	639
	R: AGTAACGGTACTTAAATTGTTTA	
*erm*C	F: TCAAAACATAATATAGATAAA	642
	R: GCTAATATTGTTTAAATCGTCAAT	
*msr*A	F: GGCACAATAAGAGTGTTTAAAGG	940
	R: AAGTTATATCATGAATAGATTGTCCTGTT	
*msr*B	F: TATGATATCCATAATAATTATCCAATC	595
	R: AAGTTATATCATGAATAGATTGTCCTGTT	
*lnu*A	F: GGTGGCTGGGGGGTAGATGTATTAACTGG	323
	R: GCTTCTTTTGAAATACATGGTATTTTTCGATC	
*lnu*B	F: CCTACCTATTGTTTGTGGAA	925
	R: ATAACGTTACTCTCCTATTC	
*lsa*A	F: GGCAATCGCTTGTGTTTTAGCG	1200
	R: GTGAATCCCATGATGTTGATACC	

MLS: macrolides, lincosamides and streptogramins; PCR: polymerase chain reaction.

Table 1. Primer sequences and PCR fragment size of tested MLS resistance genes.

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On the other hand, pleuromutilins and streptogramins A are not included in the panel of antibiotics proposed for susceptibility testing. Probably, the values of MICs to clindamycin and quinupristin-dalfopristin, which usually are not affected by L-phenotype, can be used as indicators [46].

Molecular detections of the most common genes involved in MLS_B resistance are an accurate method for phenotype determination (**Table 1**).

6. Historical background

The first report about the activity of erythromycin was confirmed in 1954 by Derek [47]; in 1964, Macleod et al. indicated that lincomycin was effective against *S. aureus* [48]. Inducible resistance to MLS antibiotics was identified in Gram-positive bacteria by Weaver and Pattee shortly after the introduction of erythromycin into clinical practice [49]. One year later, in 1965, Griffith et al. described antagonism between lincomycin and erythromycin [50]. During their study, the authors observed an antagonistic action between lincomycin and erythromycin, when the two drugs were allowed to diffuse into the same area of an agar plate seeded with a strain of *Staphylococcus* which was resistant to erythromycin but sensitive to lincomycin. Since the molecular basis of this mechanism was unknown, the authors explained the phenomenon as the result of an altered metabolism stimulated by erythromycin on erythromycin-resistant staphylococci.

In 1971, Lai et al. demonstrated altered methylation of ribosomal RNA in a erythromycin-resistant *S. aureus* strain, whereas the same study group in 1973, concluded that modification of 23S rRNA, methylation to form dimethyladenine, was responsible for the resistance to lincomycin and spiramycin in *S. aureus* [51]. Subsequently, causation has been attributed to post-transcriptional methylation of A2058 (*Escherichia coli* numbering) at the peptidyl transferase center in domain V of 23S rRNA [52]. The family of enzymes responsible for A2058 has been designed as Erm (**e**rythromycin **r**esistance **m**ethylase) with the corresponding genes designed as *erm*. To date, five different methylase genes have been described in staphylococci: *ermA*, *ermB*, *ermC*, *ermF*, *ermY* and *ermT* [21, 53–57].

In 1990, Ross et al. identified *msrA* gene, which encodes an ATP-dependent efflux pump [15]. Esterases encoded by *ereA* and *ereB*, which inactivate erythromycin by hydrolyzing the lactone ring of the macrocyclic nucleus, were identified by Quinissi and Courvalin in 1985 [27]. On the other hand, the nucleotide sequence of *lnuA* gene, which confers resistance only to lincosamides, has been determined by Bisson-Noel and Courvalin, in 1986 [31]. Inactivation of macrolides by phosphotransferases (encoded by *mphC* genes) has also been described by Wondrack et al. in 1996 [29].

To date, a variety of genes (such as *vgaA*, *vgaC*, *vgaE*, *lsaE*, *vgaA*, *lnuA*, *lnuB*, and *mphC)*, which are involved in the MLS-resistance expression, have been described and are disseminated among staphylococcal species.

7. Epidemiology of MLS_B resistant staphylococci: recent data

Staphylococcus aureus and coagulase negative Staphylococci (CONS) are challenging pathogens causing a variety of infections (minor skin and soft tissue infections, endocarditis,

pneumonia, septicemia, etc.) [58], while the emergence of drug-resistant staphylococci is an important public threat [59]. The isolation frequency of methicillin-resistant *S. aureus* (MRSA) has dramatically increased in the recent years [60]. Thus, these factors have led to a renewed interest in the use of macrolides, lincosamides and streptogramins B (MLS_B) antibiotics for the treatment of staphylococci-associated infections. From these antibiotics, clindamycin is the preferable agent, because of its excellent pharmacokinetic properties [61]. Additionally, clindamycin is the preferred agent due to its proven efficacy, low cost, the availability of its oral and parenteral forms, tolerability, excellent tissue penetration, its good accumulation in abscesses and because no renal dosing adjustments are required. Clindamycin also inhibits the production of staphylococcal toxin, and can be used as an alternative of penicillin, in patients who are allergic to the latter agent [62]. However, the widespread use of the MLS_B antibiotics has increased the number of the Staphylococcus isolates which are resistant to them [63].

The rate of MLS_B-resistant staphylococci varies between countries and species. Unfortunately, in the last decade, data concerning the rate of MLS resistance in staphylococci are limited. Otsuka et al. reported that 97% of MRSA and 34.6% of MSSA were resistant to one or more MLS_B agents in a study conducted between 2001 and 2006 [64]. Cetin et al. in a large collection of staphylococci in a Turkish hospital have found that 38.5% were resistant to MLS_B antibiotics, while Uzun et al. reported that during 2011–2012, 79% isolates were found as erythromycin-resistant in a tertiary hospital in Ismir [65, 66]. In a tertiary Greek hospital, the rate of MLS_B *S. aureus* reached to 44%, whereas in Cyprus 67.61% of *S. aureus* and 59.4% of the coagulase-negative staphylococci were resistant to erythromycin [67, 68]. On the other hand, high rate of erythromycin-resistant staphylococci was also observed in veterinary [69].

Regarding the distribution of resistant phenotypes, the most common are MLS_B (constitutive or inducible) followed by MS_B. In Japan, Otsuka et al. revealed higher incidence of the MLS_B-inducible phenotype than in Europe, Turkey and the USA [41, 64, 70–73]. Such differences in the incidence of phenotypes might reflect differences in the drug usage, the gene carriage and the clonality of strains.

Totally, 92 genes, which confer resistance to MLS antibiotics, have been described to date. They can be roughly divided into three groups, depending on the mechanisms by which they confer resistance to one or all of these groups of antibiotics. Data from different studies agree that the most prevalent genes are *ermA* and *ermC* followed by *msrA* gene [41, 70–74]. Gatermann et al. have demonstrated that in a large collection of coagulase-negative staphylococci *ermC* gene predominated and was constitutively expressed, whereas in *S. aureus* the *ermA* predominates [65, 75]. In livestock *S. aureus* strains, such as CC 398, other genes such as *ermT*, *lnuB* and *lsa* are detected [76–78]. In contrast, *mphC* gene is frequently found in staphylococci isolated from animals [79, 80].

8. Conclusions

Staphylococci and specially *S. aureus* are considered as important pathogen in a wide variety of human and animal infections. The sharp emergence and a spread of methicillin-resistant

staphylococci in the community setting and the occurrence of vancomycin-resistant staphylococci, along with vancomycin-intermediate *S. aureus* are of concern. This phenomenon has led to the development of new antimicrobial compounds. Moreover, traditional antibiotics, such as MLS_B, should be carefully considered for the treatment of infections caused by multiple drug-resistant staphylococci.

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Staphylococcus aureus strains are an important medical infectious agent that causes a wide range of pathogeneses starting from colonization of the skin and mucosal surface to severe pathogenic effects such as septicemia. The mortality and morbidity from this pathogen are challenging issues for the healthcare premises. Methicillin Resistant *Staphylococcus aureus* strains (MRSA) are causing severe infections due to the genes that are resistant to several antibiotics including methicillin, aminoglycosides, and others. Recently, there have been several reports related to failure of treatment plans caused by MRSA that led to Vancomycin Intermediate *Staphylococcus aureus* strains (VISA) or, in sporadic cases, resistance to the drug of choice.

This book highlights the new areas for the treatment of MRSA using natural products. The implementation of specific products produced by this organism can help the scientist to obtain a new window for treatments such as anticancer chemotherapy, antioxidants, etc.

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