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Review article

Resistance in Staphylococcus Aureus: The Never-Ending Story

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SUMMARY

Combating *Staphylococcus aureus* (*S. aureus*) infections using antibacterial drugs is actually an ongoing effort to overcome resistance mechanism of this microorganism. In this paper, we discussed (1) the mechanisms of resistance to some of the most commonly used antimicrobial agents in the treatment of *S. aureus*: methicillin, vancomicyn and quinolones. In addition, (2) efflux pump mechanisms involved in maintaining homeostasis in the presence of compounds that inhibit S. aureus growth and reproduction, as well as mechanisms of resistance to a number of antibiotics, have been reviewed.

Key words: Staphylococcus aureus, **resistance mechanisms, antimicrobials**

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INTRODUCTION

It is considered that drug and multidrug resistance may raise the level of virulence factors in pathogenic bacteria. The mechanisms responsible for increased antimicrobial resistances in the *Staphylococcus aureus (S. aureus)* include enzymatic inactivation of antibiotics, (β lactamases) decreased membrane permeability (methicillin resistance), alteration of binding sites (resistance to quinolones), presence of biofilms and active efflux of antimicrobials, which is included in resistance to many harmful substances including antibiotics (1).

In the early seventies of the last century, physicians were forced to abandon their belief that all bacterial infection can be cured by effective antimicrobial agents due to the fact that pathogens such as *S. aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* possess multiple antibiotic resistances. The development and increasing the number of bacterial strains resistant to antibiotics are based on a number of factors including the widespread and sometimes inappropriate use of antibiotics, extensive use of these agents as growth enhancers in animal feed and the increase in regional and international travel, which facilitated the transition of antibiotic-resistant bacteria across geographic barriers (2). An increasing number of multiresistant isolates of *S. aureus* can be seen worldwide among hospitalized patients in intensive care units and from hemocultures (3, 4). Therefore, nowadays we have fewer antibiotics for the treatment of life-threatening infections. Staphylococci are developing effective mechanisms of resistance at the same rate as the new drugs have been introduced in therapy.

New notifications of *S. aureus* isolates resistant to vancomycin opened a chemotherapeutic era with fear that effective antibiotics against this organism may no longer be available (5, 6). Considering that *S. aureus* can cause severe clinical manifestations, as well as infection in the hospital setting, the problem of its resistance is of great clinical significance. Namely, when penicillin was discovered, all tested strains of *S. aureus* were susceptible to penicillin. Today, virtually all strains of *S. aureus* express some of the mechanisms of resistance to this drug. Until now, several mecha-nisms by which *S. aureus* confers resistance to β-lactam antibiotics, quinolones and vancomycin have been discovered and clarified, which have the greatest significance for the treatment of infections caused by these strains. Furthermore, one of the major problems is the emergence of strains resistant to methicillin, the drug specially created for the treatment of *S. aureus* infection, in particular, methicillin-resistant *S. aureus* (MRSA). Staphylococci typically have one or more plasmids per cell that have different genes, mostly related to the mechanisms of resistance.

Generally, the genetic resistance can be determined at the level of chromosomes or plasmids. Staphylococci typically carry one or more plasmids, although it is mainly related to resistance mechanisms. They could be small ones that carry a single resistance determinant, slightly larger, which carry several resistance determinants and multiresistance plasmids (7).

RESISTANCE TO B-LACTAM ANTIBIOTICS

In *S. aureus* strains, resistance to β-lactams may be caused by enzymatic activity (β-lactamase), changes in penicillin binding proteins (PBP), and efflux pumps.

Beta-lactamases can be divided according to the mode of action and chemical (molecular) structure. According to the mode of operation, that is, according to the specificity of the enzyme for a substrate, β-lactamases are divided into four groups (1 to 4) (8). Molecular classification of β-lactamases is based on the sequences of their nucleotide and amino acid. Until today, four classes are known (A to D or A, B, C, D) that are correlated with the functional classification. Class A, C and D have serine in the active site, while zinc is required for the operation of class B (9).

Activity of β-lactamases

Most of the β-lactamases belong to the family of serine peptidase. These are enzymes produced by some bacteria and are responsible for resistance to β-lactam antibiotics, although they are relatively resistant to cephalosporins. Beta-lactamases breaks β-lactam ring through hydrolysis deactivating the molecule's antibacterial properties (10). The first identified type of β-lactamase was penicillin-specific penicillinase discovered before the practical application of penicillin (11).

The first group includes cephalosporinases, which belong to the molecular class C, and their performances are not inhibited by clavulanic acid. This group consists of Gram-negative bacteria's AMC enzymes (12, 13). The second group isdivided into several subgroups, while the group 3 are metalloenzymes of molecular class B, which also are not inhibited by clavulanic acid.

The second group, divisible into several subgroups, are cephalosporinases and penicillinases, both inhibited by clavulanic acid, belonging to the molecular class A with exception of the subgroup 2d that belongs to A and D classes, reflecting the original TEM and SHV genes. Due to the increasing number of β-lactamases derived from TEM and SHV β-lactamases, they are divided into two subclasses, 2a and 2b (12, 13). Subgroup 2a contains only penicillinase. Subgroup 2b contains a broad spectrum of β-lactamases that can inactivate penicillins and cephalosporins. This subgroup includes enzymes TEM-1, TEM-2, and SHV-1. Additionally, some new sub-groups were derived from this subgroup (12, 13). Subgroup 2b has an extended spectrum of action and the ability to inactivate third generation cephalosporins (ceftazidime, cefotaxime and cefpodoxime) and monobactams (aztreonam). This subgroup includes the enzymes TEM-3 to TEM-26 TEM, as well as SHV 2 to the SHV-6 (12, 13).

Subgroup 2br is resistant to inhibitors, since their activity is only reduced by the clavulanic acid. This subgroup includes enzymes TEM-30 and TEM-36 (12, 13). In the sub-group 2c fall carbenicillinase, PSE-1, PSE-3, and PSE-4 (12, 13). Subgroup 2d contains oxacillinase. 2d subgroup enzymes inactivate cloxacillin at higher rate than benzylpenicillin and certain rate of their activity was observed towards carbenicillin; clavulanic acid inhibits them poorly, and some of them belong to the ESBL. These enzymes can inactivate the oxazolylpenicillins such as oxacillin, cloxacillin, and dicloxacillin. Representatives of this group are OXA-1 to 11, the PSE-2 (OXA-2) (12,13). 2e subunit consists of cephalosporinases that can hydrolyze, and monobactams able to inhibit the clavulanic acid. These are inducible cephalosporinases produced by *Proteus vulgaris* (12, 13). Subgroup 2f is added because it includes carbapenemases originated from a serine, as opposed to those that have zinc and are included in group 3. In this group are NMC-A *Enterobacter cloacae* and SME-1 *Serratia marcescens* (12, 13).

In the group 3, there are metalloenzymes, molecular class B, not capable to inhibit the clavulanic acid. Group 3, metallo-β-lactamase, possess zinc and these are the only enzymes with metal ion (zinc) as part of active site. The metallo-β-lactamases are capable to hydrolysepenicillins, cephalosporins and carbapenems, while carbapenems can be inhibited by group 2f (with serine in active site) and also by the members of group 3 (with zinc in active site).

Group 4 consists of penicillinases and still does not belong to any molecular class (not inhibited by clavulanic acid) (12, 13).

Some β-lactamases are called extended-spectrum beta-lactamases (ESBLs). ESBLs include: β-lactamases class A TEM SHV, CTX-M β-lactamases (class D), and OXA β-lactamases (PER, VEB, GES and IBC) (14). OXA β-lactamase inhibitors have long been known as being relatively rare, but localized in plasmids with the ability to hydrolyze oxacillin and similar anti-staphylococcal penicillin. They are responsible for the emergence of resistance to ampicillin and cephalothin, and they have a pronounced hydrolytic activity against oxacillin and cloxacillin. Clavulanic acid inhibits them poorly (15).

Although not belonging to ESBLs, inhibitor-resistant β-lactamases are commonly referred as ESBLs, because they also arise from the classical TEM or SHV-types of enzymes. There are at least 19 different TEM β-lactamases of that type. TEM variants resistant to the inhibitors are insensitive to clavulanic acid and sulbactam and show resistance to the combination of amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, and ampicillin-sulbactam, though remain susceptible to the inhibition by tazobactam as well as to the combinations of the piperacillin/tazobactam. However, there are some of them resistant to these antibiotics. At present, these enzymes are mostly described in France and in several locations in Europe (16).

Gene blaZ is responsible for penicillin resistance of *S. aureus*. That gene carries the information for the synthesis of β-lactamase. This dominant extracellular enzyme, which is synthesized when exposed to staphylococcal β-lactam antibiotics, hydrolyzes the β-lactam ring, thereby inactivating it. Gen blaZ is controlled by two functionally opposed genes, blaR1 activator and bla1 repressor (17), responsible for the synthesis of regulatory proteins BlaR1 and BlaI. When exposed to β-lactam, BlaR1 works as a protease that degrades repressor BlaI, directly or indirectly, allowing blaZ gene to synthesize the enzyme (18).

METHICILLIN RESISTANCE

In all of methicillin-resistant *S. aureus* (MRSA) isolates, the gene responsible for methicillin resistance (mecA gene) is an integral part of mobile genetic elements found in all of MRSA isolates. Katayama et al. in 2000 showed that the mecA gene is a part of the sta-

phylococcal cassette chromosome mec (SCCmec) (19). Thus far, description is given for the four different parts of the genomic SCCmec elements. They vary in size from 21 to 67 kilobases (kb) (20). For the synthesis of penicillin-binding protein 2a (PBP2a,) also named the PBP2, the gene of the size of 78-kDa is responsible (21). Its activity depends on the serine protease activity from which it originated.

PBP is a membrane enzyme that catalyzes the transpeptidation necessary for the cross-linking of the peptidoglycan chain (22). In addition to innate resistance, gene mutations can lead to a reduction in the amount of PBP and decrease in the affinity of PBP to antibiotics. *S. aureus* has 'supernumerary' PBP (PBP2a or PBP2'), encoded by mecA gene located on transposon, which can be transmitted through transduction as well as conjugation. The regulation of this gene takes place at the level of transcription, therefore the detection of inducible, delayed inducible, and constitutive phenotype is possible (13).

Due to his low affinity to all antibiotics that posses β-lactam ring, PBP2a allows the survival of staphylococci exposed to high levels of these antibiotics. Accordingly, the resistance to methicillin indicates drug resistance to all β-lactam antibiotics. The soluble derivatives of PBP2a have crystal structure. Perhaps, that is why PBP2a differs from other PBPs in respect of its active site which not only blocks binding of all ß-lactams, but allows the continuation of transpeptidation reaction (23).

Each MRSA strain is resistant to certain methicillin concentration, which is characteristic „fingerprinting" reflecting in an increase in the number of bacterial cells (24). Resistance expression in some MRSA strains is controlled by genes homologous to blaZ regulatory genes. Regulatory mecI and mecRI genes regulate the response of mecAtoa β-lactam antibiotics in a similar way that blaZ genes control the response to exposure to penicillin by the blaRI and blaI genes. The fact is that DNA sequence associated with repressor genes that achieve the inhibition of the gene is identical to the active ones (25). This similarity, between mecI-mecRI and blaRI-blaI regulatory genes that leads to the induction of expression of the mecA gene causes the possible emergence of an alternative way in the occurrence of resistance. The deletion or mutation in the mecI gene, or in the mecA promoter region, often leads to constitutive expression of mec (26). It has been found that mecI or blaI must be functional in all MRSA (27): that is why authors assumed its protective role in hyperproduction of toxic proteins. In contribution to the heterogeneous methicillin resistance, key role is played by an additional series of genes, fem genes (factor essential for methicillin resistance); their primary function is in the cross-linking of peptidoglycan (28).

In mecA gene sequence, more genes responsible for antimicrobial resistance can be found, insertion sequences, and genes with an unknown function. As a part of four SCCmec sequences two recombinases, ccrA and ccrB are recognized. Interestingly, they belong to invertase/resolvase family. For that reason they are responsible for site-specific integration and excision from the chromosome. That sequence of chromosome is a part of an open reading frame. The function of that sequence is not known and it is placed in the vicinity of the origin of replication at attBscc place (29, 30).

RESISTANCE TO QUINOLONES

The origin of resistance to quinolones is in chromosomal mutations. High concentration of bacteria in one place, probably as a presence of resistant strains of the subpopulation, and sometimes, limited concentration of quinolones, stimulate the emergence of resistant mutants (31). Quinolones affects the activity of DNA gyrase, weakening the bonds of DNA chain, whereas topoisomerase IV separates DNA chains. The change in amino acid sequences, in the corresponding region of the DNA enzyme complex (quinolone resistance determining region, QRDR), reduces the affinity of the quinolones to the target sites. GrlA subunit (ParC) of topoisomerase IV and GyrA subunit of DNA gyrase are considered as the most common sites of the mutations that lead to the emergence of resistance to these drugs (31, 32). Sometimes, a single amino acid mutation is sufficient to get a clinical manifestation of the resistance, but for fluoroquinolones the occurrence of additional mutations is necessary. The mutations accumulate in QRDR region, increasing the degree of resistance. An additional mechanism of resistance in *S. aureus* is the induction of NorA efflux pump to discharge more drugs. The increasing in the expression of these pumps in *S. aureus* leads to the higher rates of resistance to quinolones (33). The bond between virulence and antimicrobial resistance is very interesting: it has been found that exposure of the strains already resistant to quinolones increases the expression of fibronectin-binding protein, the surface protein that allows the attachment of bacteria to the surface of a tissue, that is, the colonization (34).

RESISTANCE TO VANCOMYCIN

Up till now, two mechanisms of *S. aureus* resistance to vancomycin were discovered. One of the mechanisms has been identified in vancomycin intermediate *S. aureus* (VISA) strains, wherein the MIC of vancomycin varies from 8-16 μg/ml (35). The decrease of susceptibility to vancomycin appears as a result of changes in the biosynthesis of peptidoglycan. VISA strains are characterized by the appearance of irregular shapes and thickening of the cell wall, because of the additional amount of synthesized peptidoglycan. Additionally, the reduction of cross-links of peptidoglycan fibers leads to the greater exposure of D-Ala-D-Ala residues (36, 35). Modification of the cross-linking agent leads to a reduction of available amount of L-glutamine required for the amidation of D-glutamate in the peptidoglycan of the bridge (6). As a consequence, vancomycin binds to the greater number of D-Ala-D-Ala residues. The vancomycin then acts as a barrier, preventing further binding of drug molecules to the target in the cytoplasmic membrane (37). Conjugal transfer of the vanA operon of the vancomycin-resistant *Enterococcus faecalis (E. faecalis)* can lead to the resistance to vancomycin. A result of this process is probably the appearance of the VRSA strains which are completely resistant to vancomycin with MIC=128 mg/ml. Changes in the terminal of the peptide chain caused the resistance of these strains, so that, instead of D-Ala-D-Ala, there is D-Ala-D-Lac. Synthesis of D-Ala-D-Lac only occurs where exposed to low concentrations of vancomycin (38).

There are several phenotypes of vancomycin resistance: VanA, VanB or VanC. VanA phenotype leads to the synthesis of the depsipeptide and its incorporation in the cell wall instead of in D-alanyl-D-alanine. In the case of VanB phenotype, the synthesis of ligase with the new specificity occurs, hence the termini of the peptidoglycan precursor have a D-lactate. Unlike the two previous phenotypes, in VanC resistance phenotype, there is D-Ser at the end of peptidoglycan precursors (13, 39).

RESISTANCE DUE TO REDUCED PERMEABILITY OF THE OUTER MEMBRANE

Mutations that lead to reduced manifestation of, or damage porins, are responsible for reducing the susceptibility to many antibiotics (1).

RESISTANCE DUE TO ACTIVE PUMPING (ACTIVE EFFLUX) DRUG

The introduction and widespread use of antibiotics has created selective pressure for the emergence of bacterial strains that would persist despite antibiotic toxicity. Not only the accumulation of resistance genes through the process of conjugal transfer but also the participation of the class of genes that encode membrane proteins can cause the appearance of multidrug resistant strains. Products of these later genes are named multidrug transporters. Whatever transporters are into question, each expels abroad spectrum of harmful substances for the cell as well as chemically unrelated drugs. These transporters may be classified in the group of defense mechanisms. These mechanisms are not rear and they are expressed in many species (40).

Efflux transporters allow the bacteria to create an important mechanism of resistance to antibiotics, by throwing out of the cell a large number of unwanted and harmful substances (41). Based on the source of energy used, they can be divided into two basic groups. The first group includes cassettes that bind adenosine triphosphate (ATP), that is, ABC transporters that use ATP as a source of energy, while another group, secondary transporters use electronic transmembrane proton or sodium ion gradients of. Competition may be an integral feature of secondary multidrug transport (42). The secondary transporters for a larger number of drugs are divided into four superfamilies according to the structure: 'major facilitator superfamily' (MFS); 'resistance-nodulation-division' (RND), the family that owns three proteins; family transporter responsible for the discharge of a number of drugs and toxic compounds (the multidrug and toxic compound extrusion, MATE); family of small multidrug resistance transporter (small multidrug resistance, SMR) (43).

Efflux pumps in *S. aureus*

There are several multidrug and drug-specific efflux pumps well described in *S. aureus*: Qac (A, B, G, H, J), LmrS, MdeA, Nor (A, B, C, D), SdrM Multidrug Efflux Pump; TetA(K), and Tet38 (Tetracycline Efflux Pumps).

QacA and QacB multidrug efflux pumps

The best descriptions of the efflux pumps in *S. aureus* are related to QacA and QacB transporters. Members of MFS family, QacA and QacB rely on the proton motive force and expression of their genes request the presence of regulator protein QacR, which is substrate- responsive. QacA protein is plasmid-encoded, while the qacB determinant is only particularly plasmid-encoded (1). In *S. aureus* clinical isolates, the presence of the qacA and qacB is recorded in both MRSA (8%) and MSSA (3.3%) (44).

QacG, QacH, and QacJ multidrug efflux pumps

QacG, QacH, and QacJ, as multidrug efflux proteins in *S. aureus*, encoded by plasmids, with similar primary amino acid sequences, belong to the SMR family (1).

NorA, NorB, NorC, and NorD multidrug efflux pump

Most of these efflux pumps are regulated by chromosomally encoded genes, and confer the resistance to a wide variety of drugs.

NorA is also thoroughly investigated multidrug efflux pump of *S. aureus*. It can expel a wide spectrum of antimicrobial agents, among others fluoroquinolones. However, there are numerous inhibitors which inhibit the drug efflux system like reserpine, chalcone, capsaicin, and other efflux pump inhibitors:"thiopyranopyridine moiety, omeprazole derivatives, flavones, isoflavones, neohesperidosides, pentaester, spinosan A, pterocarpan, orizabin XIX, orizabin IX, epigallocatechingallate, epicatechingallate, coumarin epoxide, bergamottin epoxide, and piperidine alkaloids" (1).

NorB shares 30% and 39% aminoacid sequence similarity with efflux mechanisms of *S. aureus* NorA and QacA, respectively. NorB confers resistance not only to NorA substrates but also to some fluoroquinolones and to tetracycline (1). NorC can be negatively regulated and possess 61% amino acid seqence similarity with NorB. This pump also confers resistance to fluoroquinolones (1). NorD consists of 12 domains and there is evidence that its expression is upregulated in subcutaneous abscess. It seems that substrates of NorD are not known. Although fur is a negative regulator of norD, the restriction of free iron upregulates norD expression (1).

LmrS multidrug efflux pump

LmrS is a proton-coupled multidrug antiporter (exchanger or counter-transporter) which belongs to MFS coded by chromosomal gene of the *S. aureus*. It very efficiently extrudes drugs such as lincomycin, kanamycin, linezolid, and fusidic acid often used in *S. aureus* infection therapy (1).

MdeA multidrug efflux pump

The mdeA gene is located on chromosome and encodes a multidrug efflux pump, MdeA, and conferring resistance to some fluoroquinolones such as norfloxacin. MdeA has a 23% identity of *S. aureus* QacA. Approximately 49% of the studied *S. aureus* strains isolated from blood, which means that they are invasive strains, possess overexpressed efflux pumps. MdeA overexpressing strains are also recorded as invasive strains (1).

SdrM multidrug efflux pump

SdrM is a multidrug efflux pump which belongs to MFS. It is probably energy-dependent and regulated by MgrA (1). It shows similarity to other transporters such as NorB and QacA. Mutation in mgrA gene may lead to the greater extent of expression from sdrM (Staphylococcus drug resistance) leading to the appearance of multidrug resistant strains (1).

TetA(K) and Tet38 tetracycline efflux pump

TetA(K) is one of the plasmid-encoded MFS transporters and acts as an Na+ (K+) / H+ antiporter, showing a high level of resistance to tetracycline and some of its derivates (1). On the contrary, Tet38 determinant is encoded by chromosomal genes, and also belongs to MFS, conferring resistance to tetracycline. It is negatively regulated by MgrA, acting as SdrM efflux pump (1).

FUTURE PROSPECTS

Considering resistance, an increase in resistance rates can be expected, especially in clinical isolates, against known efficient antimicrobials, and spreading of known resistance mechanisms to susceptible strains. The appearance of new resistance mechanisms in isolates previously sensitive to antibiotics used in the treatment of invasive *S. aureus* infections represent a specific problem requesting permanent monitoring and gaining available information on the resistant strains. Perhaps accumulation of knowledge about resistance genes and efflux mechanisms are the key factors for understanding the increasing resistance in invasive *S. aureus* strains.

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Review article

Rezistencija Staphylococcus aureusa: priča koja se ne završava

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SAŽETAK

Borba protiv infekcije bakterijom *Staphylococcus aureus* (*S. aureus*) upotrebom anti-bakterijskih lekova predstavlja neprestani napor da se prevaziđu mahanizmi rezistencije kojima ovaj mikroorganizam raspolaže. U ovom radu će biti razmatrani (1) mehanizmi rezistencije prema nekim od najčešće korišćenih antibiotika u terapiji *S. aureus*: meticilinu, vankomicinu i kvinolonima; (2) mehanizmi poput efluksne pumpe, koje učestvuju u održavanju homeostaze u prisustvu jedinjenja koja nepovoljno utiču na njegov rast i razmnožavanje, kao i mehanizmima rezistencije naveći broj antibiotika.

***Ključne reči: Staphylococcus aureus*, mehanizmi rezistencije, antibiotici**