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Antibiotic resistance patterns of *Staphylococcus aureus* strains isolated from cardiovascular surgery associated infections

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ABSTRACT

Staphylococcus (S.) aureus is one the most frequently isolated pathogen in intensive care units (ICU) and a common cause of nosocomial infections, resulting in a high degree of morbidity and mortality. In the present we are confronting with the globally increasing of frequency of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in hospital associated settings and, more recently, in community settings. The knowledge of the genetic support of antibiotic resistance mechanisms in *S. aureus* strains will help us to improve the management of clinical infections in patients from hospital acquired infections. The aim of this study was to establish by using phenotypic and genotypic methods the patterns of antibiotic resistance in 37 *S. aureus* strains isolated from different clinical specimens from patients with cardiovascular surgery associated infections. The high percent (64.86%) of MRSA strains detected in this study constitutes an alarm for therapeutic options, especially among immunocompromised patients with infections associated with cardiovascular devices. The reference method to identify staphylococcal methicillin resistance is *mecA* gene detection by gene amplification, should be introduced routinely in Romanian hospitals. The genetic methods could rapidly and effectively detect MLS_B (macrolide, lincosamide and streptogramin B) and MDR (multidrug resistant) strains, becoming powerful tools in the epidemiologically studies, for monitoring the emergence of these strains in the hospital environment.

Keywords: *S. aureus, MRSA, multidrug resistance, cardiovascular surgery assosciated infections.*

1. INTRODUCTION

S. aureus is an extremely versatile human pathogen responsible for a broad range of nosocomial and community-acquired infections [1], which lead to intensive investigation of this organism over recent years. It is the causative agent of a large spectrum of human diseases, ranging from skin lesions (abscesses, impetigo) to invasive and more serious infections (osteomyelitis, septic arthritis, pneumonia, endocarditis) [2]. Its genetic plasticity has facilitated the evolution of many virulent and drug-resistant strains, presenting a major and constantly changing clinical challenge [3]. In a recent study, health care costs for all patients with *S. aureus* bacteremia in the presence of indwelling devices were high, and they were twice as high among patients with hospital acquired *S. aureus* bacteremia [4, 5]. Among these hospitalized patients with medical devices, the 12-week mortality ranged from 17% for patients with long-term indwelling catheters to 35% for patients with cardiac devices [5]. The introduction in 1959 of anti-staphylococcal semi-synthetic penicillins, oxacillin and methicillin, was followed by the emergence of methicillin-resistant *S. aureus* (MRSA). An additional

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penicillin-binding protein (PBP), a cell-wall peptidoglycan transpeptidase, named PBP2a -a PBP2 mutant with a low affinity to methicillin is responsible for methicillin resistance. PBP2a is encoded by the *mecA* gene. This gene is included in a staphylococcal cassette chromosome *mec* (SCC *mec*) which involves genes for the integration and mobility of the *mecA* gene in the bacterial MSSA host [6]. It originates by horizontal transfer and recombination from other species, such as ubiquitous *S. sciuri* or commensal *S. epidermidis* [7,8]

Hospital-acquired MRSA **(**HA-MRSA) were first identified in 1960. They evolved from five major lineages and gave pandemic clones. At first, one multilocus enzyme genotype was predominant [8,9], MRSA epidemic clones have arisen from successful epidemic MSSA strains [10]. The prevalence of MRSA has grown steadily throughout the world to reach 50% in hospitals in Japan and Spain. The HA-MRSA SCCmec elements carry various resistance genes for a large number of non-β-lactam antibiotics, aminoglycosides, fluoroquinolones, macrolides, lincosamides, tetracyclines, trimethoprim-sulfonamides, fusidic acid, or rifampin, which allow them to survive selective antibiotic pressures.

In our country there are a few studies regarding the prevalence of *S. aureus* antibiotic resistance strains in different hospitals settings, which are showing an increasing rate of antibiotic resistance especially in immuncompromised patients [11, 12]. In this study we aimed to study the antibiotic resistance patterns among patients with cardiovascular surgery associated infections in order to establish potential correlations between the source of isolation and antibiotic resistance patterns.

2. EXPERIMENTAL SECTION

2.1 Bacterial strains. The study was performed on 37 *Staphylococcus aureus* strains selected from a pool of strains isolated in 2011 from patients admitted in the Emergency Institute for Cardiovascular Diseases Prof. Dr. C.C. Iliescu, Bucharest. The analyzed strains originated from immunocompromised patients with cardiovascular surgery associated infections. 50% of the patients from whom *S. aureus* was isolated exhibited at least one cause of immunodepression, the most frequent being diabetes type II and obesity renal failure. The strains were isolated from five types of clinical specimens: broncho-pulmonary secretions (7), blood cultures (3), throat swabs (10), wound secretions (14), and vaginal secretions (3), and were identified by help of API 20 Staph (bioMerieux, Lyon, France) microtests and VITEK II automatic system. The phenotypic and genotypic methods were used for characterisation of antibiotic resistance patterns of analyzed strains. Molecular methods were used for the establishment of genetic determinism of antibiotic resistance patterns of analyzed strains isolated from nosocomial infections.

2.2. Antibiotic susceptibility testing of the analyzed *S. aureus* strains was performed using the standardized disk diffusion method (following CLSI 2011 recommendations). The following Oxoid disks were used: gentamycin (GM 10 µg), tetracycline (TE 30 µg), erythromycin (E 15µg), clindamycin (CD 2µg), teicoplanin (TEC 30 µg), ciprofloxacin (CIP 5 µg), cefoxitin (FOX 30 µg), penicillin (P 10 units), tetracycline (TE 30 µg) and trimethoprim-sulfamethoxazole (SXT 1.25/23.75 µg). According with the standard disk diffusion recommendations two reference strains were included: *Staphylococcus aureus* ATCC 25923 for disk diffusion control. The detection of methicillin resistant *S. aureus* (MRSA) phenotype was performed by testing each *S. aureus* strain by disk difussion method using cefoxitin disk. The strain resistant/susceptible to cefoxitin is reported as being resistant/susceptible to oxacillin. The strains with diameter of inhibition zone around cefoxitin disk $\leq$ 21 mm are susceptible to cefoxitin, being termed MSSA *(methicillin-susceptible S. aureus)*. The resistance to methicillin and MLS_B are the two most important resistance phenotypes encountered in this opportunistic pathogen. MLS_B resistance in staphylococci can be either

constitutive or inducible. *In vitro*, staphylococci isolates with constitutive resistance are resistant to both erythromycin (E) and clindamycin (CD) while isolates with inducible resistance are resistant to E, but appear susceptible to CD [13]. Constitutive resistance to CD can be detected by standard susceptibility testing methods, being determined by 23S rRNA methylation, which is leading to poor binding of MLS_B agents to ribosoms. *S. aureus* strains with constitutive MLSB resistance are exhibiting *in vitro* resistance to all MLS_B agents. An inducible CD resistance (MLS_Bi) phenotype is installing after a macrolide exposure. The low concentration of erytromycin is an effective inducer of inducible MLSB resistance. It will induce the production of a methylase, which allows CD resistance to be expressed. This type of resistance cannot be detected by standard disk diffussion methods. For detection of inducible CD resistance the disk diffusion induction test (D-test) has been used [13]. Erythromycin resistance can be determined by three mechanisms: (i) the use of an energy-dependent efflux, (ii) production of inactivating enzymes and (iii) target modification [14]. On the other hand, tetracycline resistance is mediated by enzyme inactivation, ribosomal protection proteins and efflux proteins [15]. In conclusion, in order to establish the mechanism responsible for resistance to these antibiotics, the genetic determinants must be detected by using molecular methods

2.3. DNA extraction. Genomic DNA was extracted from 37 clinical strains. About 10 single colonies of each strain cultured on Chapman medium was inoculated into 5 mL of BHI broth and grown overnight at 37°C with shaking at 300 rpm. From these strain cultures, DNA extraction was performed by using Wizard SV DNA Genomic Purification System kit (Promega, U.S.) in according with the manufacturefs recommendations and using lysostaphin (100 µg/ml; Sigma, Germany) to achieve bacterial lysis. DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in an Applied Biosystems 2700 Thermal Cycler.

2.4. PCR Assay. Individual PCR assays were used for detection of *mecA* gene, responsible for methicillin resistance, and of *msrA* gene, encoding for MLS_B resistance due to active efflux mechanism. A multiplex PCR was used to establish the genetic determinism of resistance to tetracycline (*tetK, tetM* genes). Finaly in order to detect resistance to MLS_B due to ribosomal target modification we used a multiplex PCR with primers for *ermA* and *ermC* genes, responsible for this phenotype. The sequences of specific primers used in PCR assays, the amplicons size, and the amplification programs used are listed in tables 1 and 2 [15-19]. The amplified products were separated in 1.5% agarose gels, and stained with ethidium bromide (10µg/ml), and detected by a UV transillumination (wavelength 312 nm). The amplified genes were identified on the basis of fragment size (shown in Table 1).

Table 1: The nucleotide sequences used in PCR assays for antibiotic resistance genes detection in analyzed *S. aureus* strains.

Gene	Primer	Nucleotide sequence	Amplicon size (bp)
ermA	ermA-F	5'-AAG CGG TAA ACC CCT CTG A-3'	190
	ermA-R	5'-TTC GCA AAT CCC TTC TCA AC-3'	
ermC	ermC-F	5'-AAT CGT CAA TTC CTG CAT GT-3'	299
	ermC-R	5'-TAA TCG TGG AAT ACG GGT TTG-3'	
tetK	tetK-F	5'-GTA GCG ACA ATA GGT AAT AGT-3'	360
	tetK-R	5'-GTA GTG ACA ATA AAC CTC CTA-3'	
tetM	tetM-F	5'-AGT GGA GCG ATT ACA GAA-3'	158
	tetM-R	5'-CAT ATG TCC TGG CGT GTC TA-3'	
mecA	mecA-F	5′-ACGAGTAGATGCTCAATATAA- 3′	293
	mecA-R	5′-CTTAGTTCTTTAGCGATTGC- 3′	
msrA	msr-F	5′-TGCAAATGGCATACTATCGTC-3′	160
	msr-R	5′-CAAGAACGCTCAAGTGCTTC-3′	

Table 2: The amplification conditions used in PCR assays for detection of antibiotic resistance genes

Gene	Amplification program					
	Initial denaturation	**No. of cycles**	**Denaturation**	**Primer annealing**	**Primer extension**	**Final extension**
ermA *ermC* *tetK* *tetM*	95°C, 2 min	30	95°C, 30 sec.	54°C, 30 sec	72°C, 30 sec.	72°C, 5 min
mecA *msrA*				55°C, 30 sec	72°C, 30 sec.	72°C, 5 min

3. RESULTS SECTION

3.1. The antibiotic resistance phenotypes detected in analyzed *S. aureus* strains. The analysis of results of disk diffusion assays showed that all analyzed strains exhibited resistance to penicillin, but were susceptible to trimethoprim-sulfamethoxazole, ciprofloxacin and gentamycin. The strains resistant to penicillin exhibited this phenotype due to the production of a β-lactamase, which is inactivating the penicillinase-labile penicillins. A number of 24 of strains exhibited MRSA phenotype, being resistant to all currently available β-lactam antimicrobial agents, with the exception of the newer cephalosporins with anti-MRSA activity. Out of the tested MRSA strains, 10 exhibited resistance to three other antibiotics from other classes, i.e.: erithromycin, clindamycin and tetracycline, being thus MDR strains. A high proportion of the analyzed strains (21) exhibited simultaneously MLS_B and MRSA phenotypes. Out of the 14 strains resistant to tetracycline, 12 were MRSA.

3.2. The genetic determinism of antibiotic resistance in *S. aureus* strains. The analysis of PCR results is showing the presence of *mecA* gene in 24 strains with MRSA phenotype, which is demonstrating that the mechanism of resistance to methicillin is due to the synthesis of an abnormal penicillin binding protein, PBP2a or PBP2', encoded by *mecA* gene (Fig. 1). It is well known that *mecA* gene complex contains insertion sites for plasmids and transposons, which facilitates the acquisition of resistance genes from other antibiotic clases. Horizontal transfer of *mecA* gene in MSSA strains represents the most important mechanism for the spread of of methicillin resistance in *S. aureus* strains. The analysis of multiplex-PCR results for detection of *ermA* and *ermC* genes, responsible for MLS_B resistance phenotype, in *S. aureus* strains showed that the strains isolated from wound secretions, throat swabs, vaginal secretions, and broncho-pulmonary secretions possess *ermC* gene, while the strains isolated from blood cultures the *ermA* gene (Fig. 2). Also, the analysis of PCR results for *msrA* gene showed the absence of this gene among *S. aureus* strains resistant to erythromycin, suggesting that the mechanism responsible for the occurrence of MLS_B phenotype is not due to an active efflux mechanism. Regarding the genetic determinism of tetracycline resistance among the analyzed strains, the results of multiplex-PCR assay showed that all 12 resistant strains exhibited *tetK* gene, while *tetM* was not detected (Fig. 3).

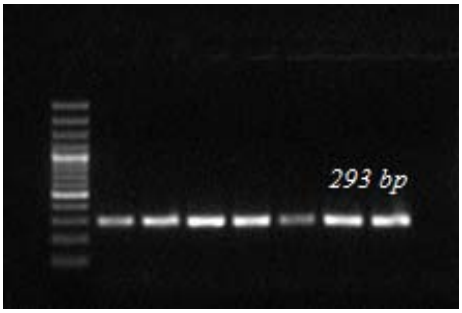

Figure 1: Gel electrophoresis of amplification products of *mecA* gene in *S. aureus* strains. M - Gene Ruler 100bp Plus DNA Ladder (Fermentas).

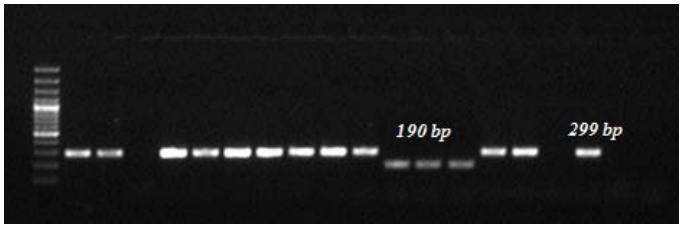

Figure 2: Gel electrophoresis of amplification products by multiplex-PCR of *ermA* and *ermC* genes in *S. aureus* strains. M - Gene Ruler 100bp Plus DNA Ladder (Fermentas).

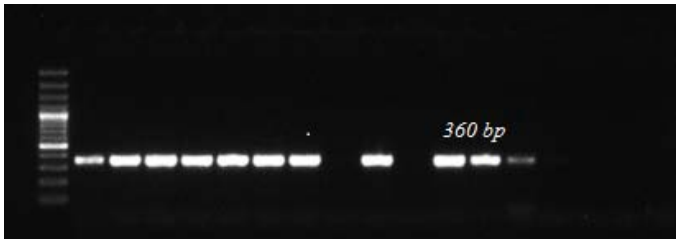

Figure 3: Gel electrophoresis of amplification products by multiplex-PCR of *tetK* and *tetM* genes in *S. aureus* strains. M - Gene Ruler 100bp Plus DNA Ladder (Fermentas).

The high number of MRSA strains (24 of 37) is confirming the increasing prevalence of MRSA strains in Romanian hospitals. Also, a previous study aimed to test the antibiotic resistance of strains isolated from patients hospitalized for systemic infection in the "Dr. V. Babes" Hospital for Infectious and Tropical Diseases during 01.01.2005-11.11.2009, showed that the average incidence of MRSA strains in systemic infections was 34.28% [20]. Another Romanian study has investigated the prevalence of this phenotype in *S. aureus* strains isolated from hospitalized patients during a period of three years (October 2005 - October 2008) in the Clinical Hospital of Infectious Diseases Iaşi, and showed a rate of methicillin resistance of 48% [21].

The results of PCR assays concerning the genetic determinism of MLS_B resistance showed the presence of *ermA* gene only in strains isolated from blood cultures, while *ermC* gene was detected in the strains from the remaining isolation sources. From the 21 strains with MLS_B phenotype, 18 had inducible MLS_B phenotype encoded by *ermC* gene, whereas 3 strains had constitutive MLS_B phenotype, encoded by *ermA* gene. Despite of the fact that MLS_B agents are widely used in the treatment of staphylococcal infections [22,23] in the recent years there were commonly reported failures during therapy with these agents [24,25].

A proportion of 27% of the MRSA strains exhibited resistance to other classes of antibiotics: erithromycin, clindamycin, tetracycline, being MDR. All analyzed strains were derived from patients with infections associated with cardiovascular tissue and prosthetic devices, which are an important cause of discomfort, disability and severe evolution leading to high mortality rates [26]. These infections involve biofilm formation, being very challenging due to resistance of bacteria from biofilm to both host immune responses and available chemotherapies [27]. As many *S. aureus* strains are becoming MDR, the search for new therapeutic approaches becames stringent.

4. CONCLUSIONS

All *S. Aurues* analyzed strains, regardless of isolation source exhibited resistance to penicillin, results that are confirming the high prevalence of this phenotype in nosocomial *S. aureus* strains. The high level of methicillin resistance of *S. aureus* strains isolated from cardiovascular surgery associated infections (64.86%) justifies a particular approach of initial anti-staphylococcal therapy. The *ermC* and *tetK* genes were the most prevalent erythromycin and tetracycline resistance determinants, respectively, in MRSA strains. The association of these resistance genes with mobile genetic elements possibly enhances the spread of resistant traits in MRSA. The rapid detection of

MRSA and MLS_B strains can be useful in epidemiologically studies and to monitor the emergence of these strains in hospitals. Also, extending these studies on a high number of strains from different isolation sources could allow the establishment of specific correlations between particular resistance genotypes and type of infection produced by *S. aureus.*

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