Microarray Technology for the Detection of Antimicrobial Resistance Genes Present in Hospital and Community Acquired Methicillin Resistant *S.aureus* Strains

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Antimicrobial resistance (AMR) represents a real burden for the modern medicine. One of the most frecvently isolated hospital acquired (HA) pathogens wordlwide, is Methicillin resistant Staphylococcus aureus (MRSA). Recently not only HA, but also community-acquired MRSA (CA-MRSA) infections have been reported. A prospective study was performed between February 2009 and October 2010, with the aim to investigate bacterial resistance of CA-MRSA and HA-MRSA. DNA microarray technology has been used for the detection of 4 AMR genes for the studied MRSA strains. A number of 218 HA- S.aureus strains have been isolated, from which 89 (40. 82%) were MRSA. In the community, 1.553 S.aureus strains were isolated, out of which, 356 (22. 92%) were MRSA. From these, a number of 17 HA and 12 CA –MRSA strains have been analyzed by DNA microarray technology. From 100% phenotypically described HA- MRSA, we identified mecA gene in 10 strains (58. 83%). Other 6 strains (35. 29%) have been erm(A) positive and 4 (23. 53%) - tet(O) positive. 83. 33% (10 strains) from the CA strains had mecA gene, only one (8. 33%) was erm(A) positive and 4 (33. 33%) were erm(C) positive. DNA microarray is a method allowing the concomitant scan of multiple genes and can be done within a few hours. That type of rapid and reliable methods for antimicrobial sensitivity tests are important to start an appropriate therapy.

Keywords: MRSA, mec A, erm(A), erm(C), tet(O), microarray

Antimicrobial resistance (AMR) represents a real burden for the modern medicine. One of the most frecvently isolated hospital acquired (HA) pathogens wordlwide is Methicillin resistant *Staphylococcus aureus* (MRSA) [1-3] .

Often, HA- MRSA strains are resistant to many other classes of antimicrobial agents, being described as multidrug-resistant (MDR) or even extensively drug-resistant (XDR), due to resistance genes encoded on mobile genetic elements, such as plasmids and transposons [3,4]. The rise of MDR/XDR- MRSA represents a real problem in terms of treatment and control [4,5].

Recently not only HA, but also community-acquired MRSA (CA-MRSA) infections have been reported. The former are contracted from schools, child care, day care centers, gyms, or prisons. Infections caused by CA-MRSA strains are a particular concern because they spread more quickly. Often, HA-MRSA strains move out into the community and CA-MRSA moves into the hospitals and there are fears that these strains will eventually replace HA-MRSA strains in healthcare settings, although Kouyos R. *et al* suggests otherwise [8].

The most important mechanism of resistance to penicillin is beta-lactamase production, which inactivates penicillin by hydrolysis of its beta-lactam ring. Another mechanism is associated with penicillin-binding protein 2a (PBP2a), encoded by *mec*A [9]. MRSA strains carry the *mec*A gene, which is part of a larger piece of foreign DNA, known as the SCC *mec* element, which is not normally found in *S. aureus*. The process of transferring DNA is known as horizontal gene transfer.

Classical antimicrobial sensitivity testing (AST) methods (like disk diffusion, broth dilution, E-testing) are rather laborious and time consuming. Although the automated systems are fast and precise, they are expensive. The genotyping tests (by PCR and/or hybridisation methods) are precise, relatively cheap and much faster (up to 24 h when performed on bacterial cultures, down to a couple of hours when performed directly in the biological product) [10]. Furthermore, these tests may provide important epidemiological data regarding the spatial/temporal distribution of the resistance genes in different pools, populations and environments [11]. DNA microarray is a method allowing the concomitant scan of multiple genes and has been described for typing resistance genes in Gram positive and Gram negative bacteria, but it has not become a commonly used diagnostic method due to prohibitive costs of reagents, machines and lack of qualified personnel [12-15].

The aim of the present study was the molecular diagnosis of MDR-MRSA by DNA microarray technology in both HA and CA – MRSA strains in Western Romania.

Experimental part

Bacterial strains collection and microbiological method

A prospective study was performed between February 2009 and October 2010, with the aim to investigate bacterial resistance of CA-MRSA, with strains provided by S.C. Bioclinica S.A. and HA-MRSA, with strains provided by the intensive care unit (ICU) of Pius Branzeu Emergency Clinical County Hospital Timisoara (PBECCHT). PBECCHT is the biggest regional county hospital from the Western Romania, with more than 1100 beds, having a 27 beds ICU, which provides healthcare assistance for both medical and surgical cases.

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Only the strains identified after at least 48 h of hospitalization were included as HA pathogens (all strains identified upon admission were discarded); in both HA and CA infections, we only included the first clinically relevant strain, in order to avoid duplication and phenotypic changes induced by antibiotic selection pressure. No age, gender, infection site or prior antibiotic use exclusion criteria were applied.

HA- *S.aureus* strains have been identified especially from bronchial aspirates, blood, urine samples, wound secretions, catheter tips. Identification was done using the VITEK 2 Compact (BioMerieux®, France) automated system with VITEK 2 GP cards. The sensitivity of bacterial strains was analysed by the microdilution method (AST cards) and interpreted by the VITEK 2 Compact System according to the minimum inhibitory concentration (MIC) breakpoints set by the National Committee on Clinical Laboratory and Standards Institute Inc. (CLSI M100-S16, 2010). The following quality control strains were used: *S.aureus* ATCC 43300 and *S.aureus* ATCC 29213.

MDR was defined as acquired resistance to at least one agent from three or more antimicrobial categories and XDR as sensitivity to a maximum two antimicrobial categories.

Genomic DNA isolation and labeling

DNA was extracted from fresh (24 h from inoculation) colonies, grown on sheep blood agar medium. The total bacterial DNA was extracted from half a loopful of bacterial cells suspended in 200 µL PBS using High Pure PCR Template Preparation Kit (Roche Applied Science, cat. no. 1179682800). The quality and concentration of DNA was determined with the NanoDrop ND1000 spectrophotometer. DNA was labelled with Alexa Fluor 3/5 by a randomly primed polymerization reaction and purified using BioPrime Total Genomic Labeling System (Invitrogen, cat. no. 18097-011) according to the manufacturer's directions.

Oligonucleotide design and microarray construction

Oligonucleotide probes were designed according to Frye [16] and represented 4 genes of the following classes of antimicrobials: β-lactam antibiotics, tetracycline and macrolides. The kit was designed to determine resistance genes for *S.aureus*. The oligonucleotides were manufactured and spotted in triplicate by ArrayIt Corporation (Sunnyvale, CA, USA) in an 18 well subarray format on standard glass slide (25 x 76 x 0.96 mm).

Hybridisation, scanning and analysis

Dye-labelled DNA was dried, re-suspended in HybIt 2 hybridisation buffer (ArrayIt Corporation, cat. no. HHS2) and applied to a specific well subarray prepared according to the manufacturer's directions. Hybridisation was performed in 3 h at 42°C. Protocols suggested by the manufacturer were used for post-hybridisation washing procedures. Microarrays were scanned with SpotLight CCD Scanner (ArrayIt Corporation, Sunnyvale, CA, USA). Images were analysed using GenePix Pro 7 (Molecular Devices, Sunnyvale, CA, USA) (fig.1)

Statistical analysis and ethics

The 6.04.version of the EPI-INFO program was used for statistical analysis. Percent values were compared by contingency tables, using the chi-squared test and Fisher correction. All the statistical tests were calculated with two extremities and the value of p statistical significance was considered at ≤ 0.05.

The study was approved by the Ethical Committee of the Victor Babes University of Medicine and Pharmacy Timisoara (No.10/11.10.2008), and by the partner hospital involved in the project.

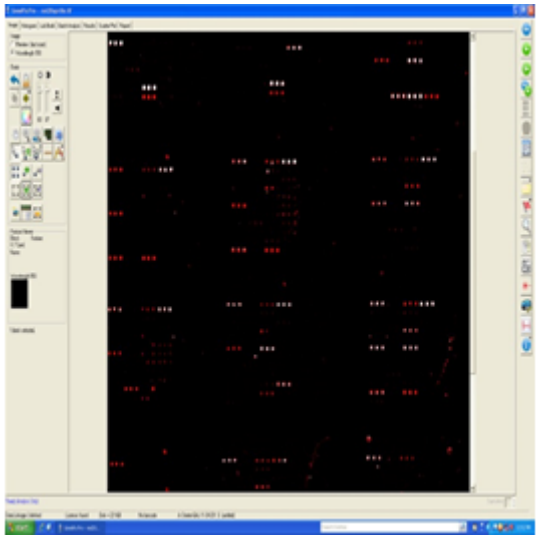

Fig. 1. Visualization of resistance genes

Results and discussions

In the studied ICU, a number of 218 *S.aureus* strains have been isolated, from which 89 (40. 82%) were MRSA. We identified 145 (66. 51%) MDR and 44(20.18%) XDR *S.aureus* strains. In the community, we isolated a number of 1.553 *S.aureus* strains, from which, 356 (22.92%) were MRSA. Also, we identified 209 (13.45%) MDR and 20 (1.27%) XDR strains.

From these, a number of 17 HA and 12 CA –MRSA strains have been analyzed by DNA microarray technology. Almost all the 17 HA – MRSA strains associated other resistance phenotypes, being included in the group of MDR microorganisms (presenting wild phenotypes of resistance to only 2-3 antimicrobial classes: oxazolidinones, fusidic acid, rifampicin). Fortunately, the 12 CA-MRSA strains were less resistant, with many other wild phenotypes of resistance, to other classes, like, fluoroquinolones, glycopeptides, sulfamides, etc. (table 1).

Regarding the DNA microarray technology, from 100% phenotypically described HA- MRSA, we identified *mec*A gene in 10 strains (58.83%), comparing with 83.33% CA (10 strains), with $p = 0.234$. In terms of erythromycin resistance, 6 HA strains (35.29%) were *erm(A)* positive, comparing with only one CA (8.33%), with $p=0.187$. Other 4 HA -MRSA strains (33.33%) were positive for *erm* (C) while, no *erm*(C) positive CA strains have been found (with statistical significance with $p= 0.020$). No tetracycline genotypic resistance was found in HA - MRSA strains, comparing with 4 CA strains (23.53%), with $p=0.121$.

A possible explanation for the small percentage of *mec*A gene identification should be eventually, the small amount of DNA. Because the culture media is inhibitor for DNA extraction, it is possible that it might not been enough for being marked with Cy3 or Cy5.

Nizami D. *et al* [8] reported a total of 16.5 per cent of *S.aureus* isolates showing resistance to methicillin and carrying *mec*A gene. Also, a total of 145 isolates were resistant to erythromycin, and contained at least one of the erythromycin resistance genes *erm*(A), *erm*(B), *erm*(C) *and msr*(A). The *erm*(A) and *erm*(C) genes have been detected in 77 isolates and *erm*(B) in 13 isolates. Eleven isolates carried both *erm*(A) and *erm*(B). A total of 121 isolates were resistant to tetracycline and carried either *tet(K)* or *tet(M)* or both resistance genes.

Table 1

HA AND CA - MRSA STRAINS: PHENOTYPE - GENOTYPE CORRELATION

No.	Provenience	Resistance phenotype	*mec*A	*tet*O	*erm*A	*erm*C
0	Contol strain *S.aureus* ATCC 43300	MRSA - positive control	+	-	+	-
0	Contol strain *S.aureus* ATCC 29213	*S.aures* wild strain - negative control	-	-	-	-
1	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + wild glycopeptide + constitutive MLSB +wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistante (eflux TET K) + fosfomycine, rifampicine - R	-	-	-	-
2	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + Hetero-VISA/wild glycopeptide + constitutive MLSB + wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + fosfomycine, rifampicine - R	-	-	-	-
3	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + Hetero-VISA/ wild glicopeptide + constitutive MLSB + wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + fosfomycine, rifampicine - R	+	+	+	-
4	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + wild glycopeptide + constitutive MLSB +wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + fosfomycine, rifampicine - R	-	-	+	-
5	HA	MRSA + beta-lactamases production + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + wild glycopeptide + constitutive MLSB +wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + fosfomycine, rifampicine - R	+	-	-	-
6	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + wild glycopeptide + constitutive MLSB +wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + fosfomycine, rifampicine - R	+	-	+	-
7	HA	MRSA + beta-lactamases production + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + wild glycopeptide + constitutive MLSB +wild oxazolidinone + wild - fusidic acid, SXT; Tetracycline + fosfomycine, rifampicine - R	-	-	-	-
8	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialyl resistant Fq + wild glycopeptide + constitutive MLSB +wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + fosfomycine, rifampicine – R	+	+	+	-
9	HA	MRSA + PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4"))/wild + wild Fq + wild glicopeptyde + inducible MLSB/ resistant (eflux) + wild oxazolidinone + wild - fusidic acid, SXT, fosfomycine, rifampicine + Tetracycline – target modification (TET M), partialy resistant (eflux TET K)	+	-	-	-
10	HA	MRSA + beta-lactamases production + Resistant (APH (3')- III)/Resistant (ANT (4')(4"))/wild + wild Fq + wild glicopeptyde + inducible MLSB /resistant (eflux) + wild oxazolidinone + wild - fusidic acid, SXT, fosfomicine, rifampicine+ Tetracycline – target modification (TET M), partialy resistante (eflux TET K)	+	-	-	-
11	HA	MRSA	-	-	-	-
12	HA	MRSA + PBP Modification + wild AMG + Resistant Fq + wild glycopeptide + inducible MLSB /resistant (eflux) + wild oxazolidinone + wild - fosfomicine, fusidic acid, SXT + Tetracycline – target modification (TET M), partialy resistant (eflux TET K) + rifampicine –R	+	+	+	-
13	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + wild Fq + wild glycopeptide + inducible MLSB /resistant (eflux) + wild oxazolidinone + wild - fosfomycine, acid fusidic, rifampicine, SXT, Tetracyicline	-	-	-	-
14	HA	MRSA + PBP Modification + Heterogen (APH (2")+ AAC(6')) + resistant/partialy resistant Fq + wild glycopeptide + constitutive MLSB + wild oxazolidinone + wild - fusidic acid, SXT; + Tetracycline - target modification (TET M), partialy resistant (eflux TET K) + fosfomicine, rifampicine - resistant	+	-	-	-
15	HA	PBP Modification (mecA) + Heterogen (APH (2")+ AAC(6')) + resistant /partialy resistant Fq + wild glycopeptide + streptogramine resistant (SGA-SGB)/wild + wild oxazolidinone + wild - fusidic acid + wild/R-Fosfomicine + resistant -rifampicina, resistant/wild – SXT + Tetracyline - target modification (TET M), partialy resistant (eflux TET K)	+	+	+	-
16	HA	MRSA + PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4"))/wild + partialy resistant/wild Fq + wild glycopeptide + wild MLS + wild oxazolidinone + wild - fosfomicine, fusidic acid, rifampicine, Tetracycline + R – SXT	+	-	-	-
17	HA	MRSA + PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4"))/wild + wild Fq + wild glycopeptide + nducible MLSB i /resistant (eflux) + wild oxazolidinone + wild - fosfomicina, fusidic acid, rifampicine, SXT + Tetracycline+ target modification (TET M), partialy resistant (eflux TET K)	-	-	-	-

18	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glycopeptide + streptogramine resistant (SGA-SGB)/wild+ wild oxazolidinone + wild - rifampicine, SXT + Tetracycline - target modification (TET M)/partialy resistant (eflux TET K),	+	-	-	-
19	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild+ wild Fq + wild glycopeptide + inducibe MLSB + wild oxazolidinone + wild - rifampicine, SXT +Tetracycline - target modification (TET M), partialy resistant (eflux TET K),	+	-	-	+
20	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glycopeptide + streptogramine resistant (SGA-SGB)/wild + wild - rifampicine;oxazolidone, SXT	-	-	-	-
21	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild+ resistant/partialy resistant Fq + wild glycopeptide + constitutive MLSB + wild - rifampicine;Tetracycline ;oxazolidone, SXT	-	-	-	-
22	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glicopeptide + resistant streptogramine (SGA-SGB)/wild + wild - rifampicine ; oxazolidone, SXT	+	-	-	-
23	CA	PBP Modification + Heterogen (APH (2'')+ AAC(6')) + resistant/partialy I resistant Fq + wild glycopeptide + constitutiv MLSB+ SA, wild – oxazolidone + Tetracycline - target modification (TET M), partialy resistant(eflux TET K) + resistant- SXT, rifampicine	+	-	+	-
24	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glycopeptide + streptogramine resistant (SGA-SGB)/wild+ wild - rifampicine;Tetracycline ; oxazolidone, SXT	+	-	-	-
25	CA	PBP Modification + Heterogen(AAC(2''))+AAC(6'')) + wild Fq + wild glicopeptide + inducible MLSB + wild - rifampicine, SXT +Tetracycline - target modification (TET M)/partialy resistant (eflux TET K),	+	-	-	+
26	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild+ wild Fq + wild glycopeptide + nducible MLSB + wild - rifampicine; oxazolidone, SXT + Tetracycline - target modification (TET M), partialy resistant(eflux TET K)	+	-	-	+
27	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glycopeptide + inducible MLSB + wild- rifampicine, SXT;oxazolidone + Tetracycline target modification (TET M), partialy resistant(eflux TET K)	+	-	-	+
28	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glycopeptide + streptogramine resistant (SGA-SGB)/wild+ wild - rifampicine, SXT;Tetracycline ;oxazolidone	+	-	-	-
29	CA	PBP Modification + Resistant (APH (3')- III)/Resistant (ANT (4')(4''))/wild + wild Fq + wild glycopeptide + wild MLS + wild– oxazolidone + wild- rifampicine, SXT + Tetracycline - target modification (TET M)/partialy resistant (eflux TET K)	+	-	-	-

In another study performed by Elhassan M. *et al,* 90.2% from all the MRSA studied strains were *mec*A positive, while the remaining 9.8% failed to produce the band of 310 bp specific for *mec*A gene. Bacterial DNA was isolated with the aid of ready kit from Thermo Scientific GeneJET Genomic, Lithuania [17].

Lim et al [18] reported that the *erm*(A) gene was more prevalent than the other erythromycin resistance genes in *S. aureus* isolates, and *erm*(C) gene was found mostly in coagulase negative staphylococcus (CoNS). Similarly, in a study performed by Martineau et al [19], the *erm*(C) gene has been reported to be more prevalent in CoNS.

In our study, more *erm*(A) genes were isolated in the hospital and *erm*(C) in the community.

In another study conducted by Spence R. et al, characterization of 43 *S. aureus* isolates by the microarray technology and pulsed-field gel electrophoresis demonstrated the ability of the array to differentiate between isolates representative of a spectrum of *S. aureus* types, including methicillin-susceptible, methicillin-resistant, community-acquired, and vancomycin-resistant *S. aureus*, and to simultaneously detect clinically relevant virulence determinants. The microarray technology was comprising 84 gene targets, including species-specific, antibiotic resistance, toxin, and other virulence-associated genes, capable of examining 13 different isolates simultaneously [20].

Conclusions

Our findings indicate a high prevalence of HA and also CA – MRSA, which represents a priority in terms of treatment and control. That's why, rapid and reliable tests are important, to start an appropriate therapy. If MRSA strains identification and AST by conventional methods require a minimum of 48 hours, the detection of AMR genes by DNA microarray technology can be done within a few hours.

However, the absence of *mec*A gene in a considerable percentage of MRSA isolates requires technique improvement and also, more ooligonucleotides probes needed to be designed for the identification of other antimicrobial resistance genes, by microarray technology.

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