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Evaluation of diagnostic methods in infection with *Staphylococcus* genus strains

PhD Thesis Summary

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Keywords: bacteria, antibiotic resistance, *Staphylococcus aureus*, coagulase negative staphylococcus, PCR.

INTRODUCTION

Nosocomial infections cause serious problems in most countries. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most incriminated germs.

In order to conduct appropriate treatment a rapid and accurate identification of these methicillin-resistant strains is required. There are of course traditional methods to determine susceptibility to methicillin, but the phenotypic expression of this resistance often shows a heterogeneous nature.

The use of nucleic acids as biological samples has become frequent in recent years. There are a variety of DNA samples used in routine diagnostic laboratories for confirmation of cultures and for direct detection of pathogens in biological samples.

PCR techniques have gained wide use, they showed great sensitivity and specificity [Jakab, 2009]. Conventional PCR methods use agarose gel electrophoresis to detect amplification products, are not well suited for rapid screening because of the limited number of samples which it can be analyzed on the gel, for loading there are needed additional steps, running gels and analysis. Thus, we believe that real-time PCR methods are more appropriate for these determinations.

Exclusive use of these molecular tests can lead to false positive results because not all strains possessing the *mecA* gene are able to express its product [Miller et all, 2005].

1. *STAPHYLOCOCCUS* GENUS

In 1878, Robert Koch first observed the presence of micrococcus in pus. Two years later, Louis Pasteur isolated microorganism but the name dates from 1882, when the surgeon Ogston refers to "a loop-forming clusters".

Bacteria of the genus *Staphylococcus* are gram-positive cocci with the mean diameter of 0.80 μm, aerobic and facultative anaerobic, some microaerophilic, immobile, in Gram-stained smear dropping intense gentian violet and appear as grouped in grape-like clusters, hence the genus name (Gr.staphylo = bunch of grapes). Cell wall with a strong antigen character has a complex structure consist of teichoic acids, peptidoglycan and an proteic outer layer. Cell membrane has a complex structure containing glycolipid, phospholipids and cardiolipin. Nuclear region is represented by a single DNA molecule associated with RNA and proteins [Buiuc and Neguț, 2008].

The 2004 edition of the Bergey Manual of Systematic Bacteriology, *Staphylococcaceae* family is taxonomic assigned as the eighth family of the Order *Bacillales*, Class III, *Bacilli*, belonging to the *Phylum* BXIII, *Firmicutes*, Domain *Bacteria*.

Corresponding to the main mechanisms of pathogenity, staphylococci can cause two major types of pathological manifestations: one with invasive nature, the other with a toxic nature. Invasive type of infectious may apper in case of foliculits, hidrosadenitis, mastitis, wound infection, staphylococcus angina, cystitis, urethritis, prostatitis, pyelonephritis, blood dissemination, etc. Staphylococcal toxiinfections include: food poisoning, toxic injuries and toxic shock syndrome.

An important feature of staphylococci is antibiotic resistance. Antibiotics are substances with specific action on various microorganisms. Action may be a bactericidal or bacteriostatic. Antibiotic action mechanisms consist of inhibition of protein synthesis, blocking DNA repair and replication or intervention in cell wall biosynthesis (for β-lactams). There exists antibiotic resistance mechanisms: inactivation by altering the active center of the antibiotic, modification of membrane permeability and transport, modification of antibiotic target (for methicillin-resistance *Staphylococcus* genus).

β-lactam resistance phenotypes are due to multiple mechanisms. The most common is the macromolecular target change mechanism. In this case the bacteria produce a modified

type of penicillin binding protein, called PBP 2a (PBP 2'), which has a low affinity to all β-lactam antibiotics. This modified PBP is encoded by the chromosomal *mecA* gene, which is a part of a supplementary DNA fragment integrated into the bacterial DNA. Currently three non-*mecA* related oxacillin-resistance mechanisms are described. The borderline resistant *S. aureus* (BORSA) strains are β-lactamase overproducers and they lack intrinsic resistance mechanisms (did not produce PBP 2a). In the absence of *mecA* gene some strains produce a predicted methicillinase enzyme which could hydrolyse methicillin, but its gene were not yet described. Modification of other penicillin binding proteins leads to modificated *S. aureus* (MODSA) strains. These strains have low level resistance to oxacilin and did not produce β-lactamase [Jehl, 2003].

The SCCmec (staphylococcal cassette chromosome *mec*) is an antibiotic resistance island, and is one of the largest mobile genetic elements beeing around 60 kbp [Ito et al, 1999; Katayama et al, 2000]. The SCC*mec* element contains the *mec* gene complex (the *mecA* gene and its regulatory genes, *mecI* and *mecR1*) and the *ccr* gene complex (*ccrA* and *ccrB*), encoding two site-specific recombinases responsible for the translocation of the genetic element. The SCC*mec* contain also the *Tn554* transposon and a copy of pUB110 plasmid flanked by two *IS431* insertion sequences

There are several types of *mec* complex: type A (1) show the *mecA* gene and *mecR1* and *mecI* regulatory genes and type B (2), C (3) or D (4) where *mecI* gene is missing and the gene mecR1 have deletions of some parts [Suzuki et al., 1993].

2. AIMS OF THIS STUDY

The aims of this study are:

- Evaluation of diagnostic methods in infection with *Staphylococcus* strains*;*
- testing the rapid diagnostic multiplex PCR method *mecA/nucA* for the 125 *Staphylococcus* strains;
- determination of the oxacillin minimum inhibitory concentration for the found methicillin-resistant strains;
- identification using biochemical tests of coagulase negative methicillin-resistant staphylococcal species;
- amplification of regulatory genes from *mec* region (*mecI, mecR*$_1$);
- determination of *mecI* gene sequences in order to identify possible mutations.

3. MATERIALS AND METHODS

Bacterial strains were isolated in Sibiu between July.2008 and January 2010. In the present study we used reference strain *Staphylococcus aureus* ATCC 25923 Microbiologics (Minnesota, USA).

Most commonly culture medium used in the experiments is MHA (Fluka AG, Buchs, Switzerland). In the subsequent isolation of DNA we used 5 ml of bacterial cultures grown on MHB for each interest strains (Fluka AG, Buchs, Switzerland). For MIC determinations cation adjusted MHB (2% NaCl) was used . The microorganisms were incubated aerobically at 35-37 °C for 18-24 hours.

The laboratory diagnostic in the classical system is based on a minimum identification scheme of staphylococci isolated more frequently from humans infectious (Staphylococcal National Reference Center). For the differentiation of the *Micrococcaceae* family from *Streptococcaceae* family, catalase test was used. Highlighting the catalase activity is achieved by bringing the studied culture in contact with a drop of diluted hydrogen peroxide (20%). The positive reaction is represented by the appearance of gas bubbles and differentiate Gram positive cocci from *Micrococcaceae* family from the *Streptococcaceae* family.

The *Staphylococcus* genus can be differentiated from other *Micrococcaceae* by testing its ability to metabolize glucose in anaerobic conditions, present at staphylococci and absent at micrococci. For the current needs of the laboratory diagnostic is important to identify *Staphylococcus aureus* from others coagulase-negative species using coagulase test.

Final identification of other species of medical interest can be achieved in different ways. In this study these strains were processed using the commercial API Staph (bioMérieux SA, Lyon, France) biochemical test system. The commercial kit contain 19 tests and it can be easily interpreted within 24 hours based on the Kloos and Schleifer's system. The used method is a combination of biochemical and fermentation tests, reference tests for identification of staphylococci in conjunction with a suitable database [Baldellon and Megraud, 1985]. The reliability of this system is guaranteed by using a low bacterial concentration (0.5 McFarland) inoculum, avoiding mixed cultures and subcultures. API STAPH consists by a strip containing dehydrated test substrates in individual microtubes. The tests are reconstituted by adding to each tube an aliquot of API STAPH Medium that has been inoculated with the strain to be studied. The strip is then incubated for 18-24 hours at 35-37°C after which the results are read

and interpreted. In this study the identification was made using the version 4.0 of the identification software (Fig. 1).

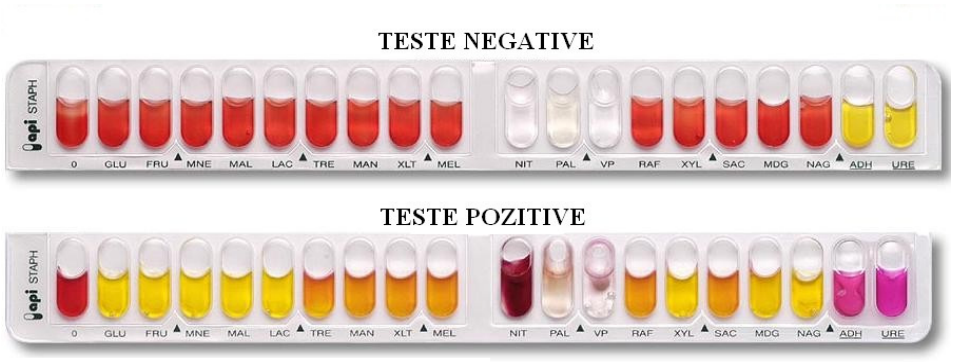

Figure. 1. Negative and positive tests on the API Staph galleries.

In this study a few staphylococcal isolates come from blood cultures. They were isolated in special conditions using automated systems. The VITEK® 2 COMPACT automated system performs within few hours the identification and antibiotic susceptibility testing of the germs. Colorimetric comparison of reading cards allows identification of bacterial strains; the turbidimetric method is used for antibiotic susceptibility testing. In the presence of antimicrobial agents the system evaluates the growth pattern for each microorganism, comparing them with a control growth-well. It uses several parameters based on observed growth characteristics to ensure the adequate data for MIC (minimum inhibitory concentration) determination. MIC results must be associated with the identification data for a correct diagnosys [Ligozzi et al, 2002]. Advanced Expert System (AES) was used for analysis, data management and also for validation of results. Using this system were obtained antibiotic resistance patterns indicating specific phenotypes [Colcieru et al, 2010].

The oxacillin/methicillin MIC's were determined using the microdiluton method (100 µl in each well). A dilution series from 0.125 to 256 µg / ml of oxacillin were used. Concentration values of the first well in which total growth inhibition will be considered MIC [ESCMID, 2003].

For isolation and purification of DNA we used the following lysis buffer: 0.2 mg/ml lizostaphin, 20 mM Tris/HCl, 2 mM EDTA, 1% Triton X-100 at pH 8.. Later, proteinase K were added to the mixture and the DNA was purified using Nucleospin® Tissue kit (Macherey-Nagel, Düren, Germany). In subsequent phases of the study for agarose gel purification of amplification products we used the NucleoSpin ® Exract II purification kit (Macherey-Nagel, Düren, Germany), All centrifugation steps were performed in BIOSAN COMBISPIN FVL and QUALITRON 2400N centrifuges.

Concentration and purity of DNA was determined using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

The duplex PCR method allows the simultaneous amplification of *mecA* gene (methicillin resistance) and *nucA* gene (a termonuclease characteristic for *S. aureus*). Oligonucleotide primers used for amplification for *mecA* gene were*:* mecA $_1$ -5 '-AAAATCGATGGTAAAGGTTGGC corresponded to nucleotides 1282 to 1303 and mecA $_2$ -5'-AGTTCTGCAGTACCGGATTTGC being complementary to nucleotides 1793 to 1814, generating a 533 bp long fragment [Murakami et al, 1991]. In case of the *nucA* gene the sequences of the two 21 and 24 base primers were: nucA $_1$ -5 '-GCGATTGATGGTGATACGGTT and nucA $_2$ -5'-AGCCAAGCCTTGACGAACTAAAGC. Primers flanks a 447 base pair long sequence of the *nucA* gene which encodes the thermonuclease A. The primer 1 bounds to 49 and 69 nucleotides, and the primer 2 bounds to 304 and 327 nucleotides. Thus a 279 pb long fragment was generated [Brakstad et al., 1992]. The oligonucleotide primers were supplied by the Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary.

For amplification of *mecI* gene (encodes MecI transcriptional repressor protein) we used MecI $_1$-5´-GAAATGGAGTAAATATAATGG primer located between positions 48877-48897 and MecI $_2$-5´-CTATGTATTGTTGTAAACAC located between positions 49292-49273, reported to the complete seqence of the *Staphylococcus aureus ssp. aureus* N513 strain (GenBank accession number BA000018). Thus a 416 bp fragment was amplified. For amplification of *mecR1* gene whichencodes MecR1 receptor protein we used two oligonucleotide preimers: MecR1 $_1$-5´-TATTACAAATGTAGTATTTATGTC located between nucleotide positions 47073-47796 and MecR1 $_2$-5´-ATTTCTTCTATTATATTATTCGCA located between nucleotide positions 48994-48971, of the same sequence; a 1922 bp fragment was generated. Both pairs of primers were synthesized at Prooligo (Paris, France) and Eurogentec (Liege, Belgium).

For duplex PCR amplification the following PCR mix was used: 5x concentrated Green reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP (from each deoxiriobonucleotide-triphosphate), 1 µM $NucA_1$ primer, 1 µM $NucA_2$ primer, 1 µM $MecA_1$ primer, 1 µM $MecA_2$ primer, GoTaq® Flexi DNA polymerase 1,25 U, 10 µl bacterial suspension or 100 ng purified DNA, and ultrapure/UV water (Purelab Ultra Genetic, ELGA LabWater, High Wycombe, Marea Britanie) up to 50 µl final volume. The DNA polymerase, reaction buffer and magnesium chloride were provided in GoTaq® Flexi DNA polymerase kit (Promega Corporation, Madison, USA). The dNTP mix was purchased from the same producer. The

samples were amplified using PCR Mastercycler® and Mastercycler® EP gradient S thermocyclers (Eppendorf, Hamburg, Germania). The used thermal profile was: initial denaturation for 5 min. at 94°C, denaturation for 30 seconds at 92°C, annealing for 30 seconds at 56°C, elongation for 90 seconds at 72°C, 5 min. at 72°C. Steps 2-4 were 30-fold repeated [Jakab and Popescu, 2005].

For amplification of *mecI* and *mecR1* gene, reaction mixture were similar, except the concentration of specific primers MecI $_1$, MecI $_2$, MecR1 $_1$, MecR1 2 (0,5 µM). The samples were amplified using Palm Cycler™ (Corbett Life Science, Sydney, Australia). The used thermal profile for *mecI* gene was: initial denaturation for 5 minute at 94°C, denaturation for 30 seconds at 92°C, annealing for 30 seconds at 53°C, elongation for 60 seconds at 72°C, 10 min. at 72°C. Steps 2-4 were 35-fold repeated. For *mecR1* gene thermal profile was: initial denaturation for 5 minute at 94°C, denaturation for 60 seconds at 92°C, annealing for 60 seconds at 56°C, elongation for 120 seconds at 72°C, 10 min. at 72°C. Steps 2-4 were 35-fold repeated.

For the separation of the amplified fragments CONSORT H_1-SET electrophoresis cell (distance between electrodes: 10 cm) and CONSORT H_U-10 electrophoresis cell (distance between electrodes: 15 cm) were used with a Consort E 835 power supply at 52 V or 80 V, respectively. We used 50 ml or 90 ml 1% agarose gel. Wells were loaded with 10 µl mixture amplified with molecular weight markers Generuler™ 100 pb DNA Ladder (Fermentas, Vilnius, Lithuania) or 1 kb DNA Ladder (Axigen Biosciences, Union City, USA). The image was captured on a DOC-IT GEL IMAGING SYSTEM (UPV, Cambridge, UK).

The mecI gene was sequenced using the Sanger method in an ABI 3730XL automated system. Samples were processed by Macrogen Inc. (Seoul, Korea). The reaction requires a minimum of 25 µl of DNA solution with a concentration of 50 ng/µl. MecI 2 primer was used at a concentration of 10 pmol/ µl (10 µM).

4. RESULTS AND DISCUSSIONS

4.1. Results of identification by classical methods

During this study a total of 125 *Staphylococcus* strains were isolated: 64 strains (51,20%) were isolated from male patients and the rest of 61 strains (48,80%) from women. The calculated average age was 57.07 years.

We can affirm that elderly people are more susceptible for staphylococcal infections, possibly due to gradual decrease of immunity with age.

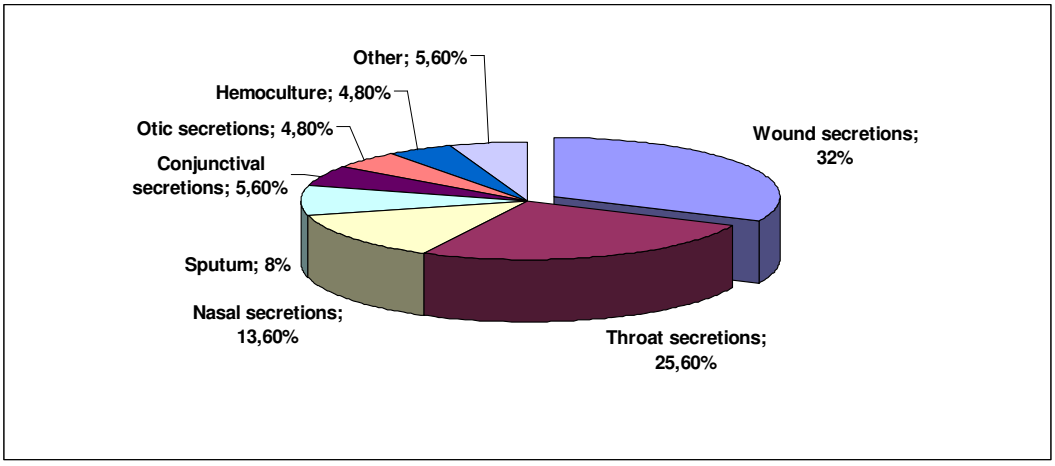

Figure 2. Share of isolated staphylococcal strains in function of pathologycal product.

In the studied samples staphylococci are present in great number in wound secretions. It can be concluded that strains of *Staphylococcus* genus are frequently isolated from wound secretions and thus involved in nosocomial infections.

All the 125 bacterial strains were processed using conventional methods: bacterial culture, biochemical tests and antibiotics susceptibility tests. There were identified 50 strains belonging to *Staphylococcus aureus* species and 75 strains of coagulase negative staphylococci (CNS).

The 125 staphylococcal strains identified by classical methods were tested by duplex PCR method to verify resistance to methicillin and confirm the identity of *Staphylococcus aureus*.

4.2. Duplex PCR method *mecA /nucA*

Duplex PCR method *mecA /nucA* a was applied to the 125 strains, and 21.60% of them were *mecA* positive, 38.40% of all tested strains were positive for the *nucA* gene and are thus was cosidered strains of *Staphylococcus aureus* (Table 1).

Tabele 1. Number of strains and their share according to the presence or absence of *mecA / nucA* gene..

Nr.	Feature	Nr. of strains	Share %
1	$mecA^+$	27	21,60
2	$mecA^-$	98	78,40
3	$nucA^+$	48	38,40
4	$nucA^-$	77	61,60
5	$mecA^+nucA^+$	13 (MRSA)	10,40
6	$mecA^+nucA^-$	14 (MRSS)	11,20
7	$mecA^-nucA^+$	35 (MSSA)	28,00
8	$nucA^-nucA^-$	63 (MSSS)	50,40

Initial evaluation of strains made by multiplex PCR method *mecA/nucA* is shown in the Figure 3. Biological sample was prepared suspending one bacterial colony in 10 µl pure water.

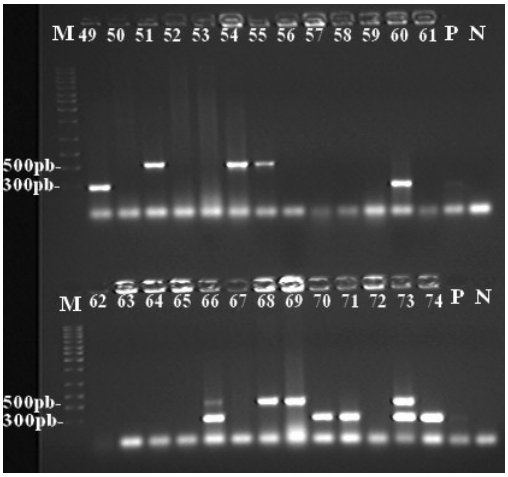

Figure 3. Results of duplex PCR amplification of *mecA /nucA* genes for strains 49 to 74.

Identifications made by direct culture have created some suspicions about the efficiency of amplification reaction in conditions where in the reaction mixture were present cellular debries and perhaps other inhibitors of the amplification reaction. After testing all strains we select methicillin-resistant strains and those who have doubts: 1, 8, 9, 15, 20, 25,

26, 27, 33, 47, 51 , 54, 55, 60, 66, 68, 69, 89, 92, 96, 97, 99, 106, 107, 111, 116, 117, 119, 124 and we isolate and purify DNA for recheck using the same method (Figure 4, 5 and 6). However strains 73, 75, 77, likely to be MRSA, were lost during the first passage.

The strain no. 47 is confirmed to be MRSA. Both the amplification products of *mecA* (533 bp) and the *nucA* genes (279 bp) were present . Another strain of interest was strain no. 25. In the case of this strain the *mecA* gene was not detected after the first PCR. Finally we detected a band corresponding to the *mecA* gene, but it was not as intense as in the case of the strain no. 47. Strain no. 25 shows a heterogeneous methicillin-resistance. Strain no. 66 is alsoconfirmed to be MRSA. In the case of the remaining strains (1, 8, 9, 15, 20, 26, 33, 51, 54, 55, 68, 69, 89) the expected amplification fragment of the *mecA* gene can be observed and they are considered MRSS [Colcieru et al., 2010].

In Figure 6 it predominates MRSA strains no. 96, 99, 106, 107, 111, 116 and 117. For strains no. 99, 106, 107, 111 and 116 it was not originally reported amplification of fragment about 533 bp corresponding to *mecA* gene. Samples 97, 119 and 124 are represented by MSSA isolates.

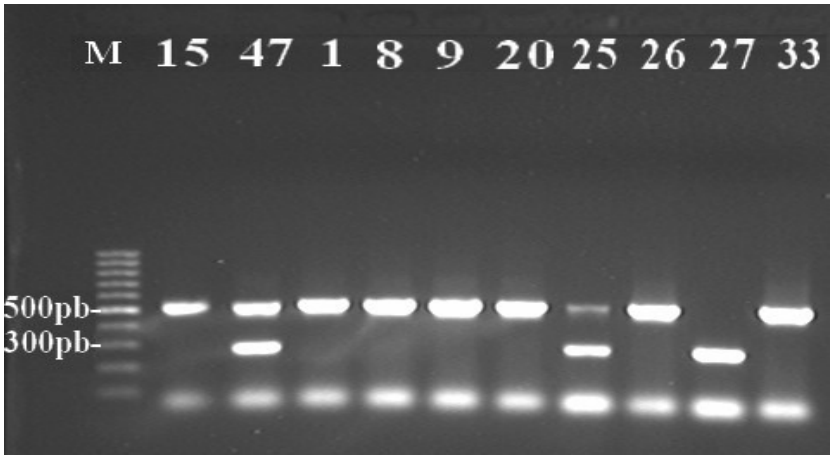

Figure 4. Agarose gel electrophoresis of amplification products after DNA isolation for strains no. 1, 8, 9, 15, 20, 25, 26, 27, 33 and 47; the unused primers form a strong band and can be observed at the bottom of the figure.

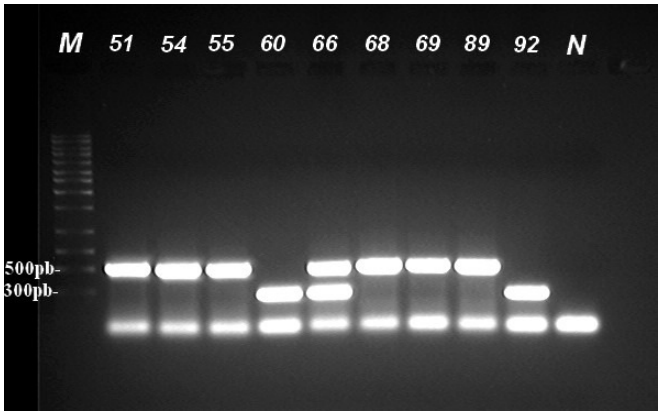

Figure 5. Agarose gel electrophoresis of amplification products after DNA isolation for strains no. 51, 54, 55, 60, 66, 68, 69, 89, and 92; on the last position you can see the presence of negative control (N).

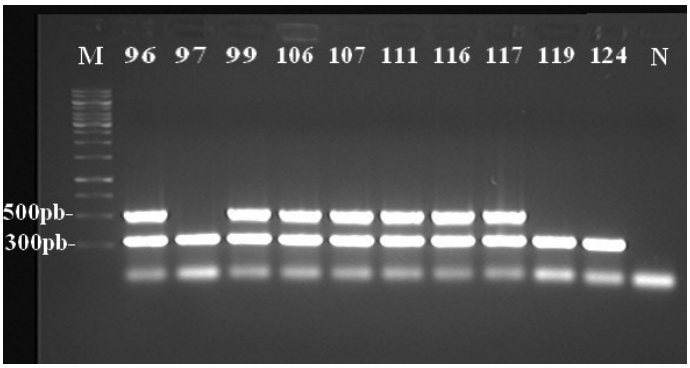

Figure 6. Agarose gel electrophoresis of amplification products after DNA isolation for strains no. 96, 97, 99, 106, 107, 111, 116, 117, 119 and 124; on the last position you can see the presence of negative control (N).

4.3. Identification of coagulase negative species using API Staph commercial system

Ten of the 23 methicillin-resistant strains identified by molecular techniques showed amplification for the 270 bp fragment specific for *Staphylococcus aureus*.

A total of 13 methicillin-resistant, coagulase-negative staphylococcus strains were identified using this commercial system. The resulted biochemical profiles were validated by using the API Web software (ver. 4.0). The most similar profiles were used for identification.

Processing reference strain Staphylococcus aureus ATCC 25932 a profile number was generated that was translated by an index value of 90.30% ID, which corespond to proper identification. The translated numerical biochemical profiles, along with identification shares for each strain are presented in Table 2.

Table 2. The identified staphylococcus strains and their similarity to the standard profile.

Isolate	Strain	Origin of the sample	Numerical profile	ID %
1	*S. warneri*	Wound secretion	6734113	49,70
8	*S. xylosus*	Conjunctival secretion	6735112	91,70
9	*S. haemolyticus*	Wound secretion	2635051	97,70
15	*S. haemolyticus*	Conjunctival secretion	6733051	82,70
20	*S. xylosus*	Conjunctival secretion	6372450	94,70
26	*S. xylosus*	Wound secretion	6372400	80,40
33	*S. haemolyticus*	Nasal secretion	6636051	47,10
51	*S. epidermidis*	Nasal secretion	6706011	93,20
54	*S. epidermidis*	Conjunctival secretion	6706010	91,10
55	*S. hominis*	Otic secretion	6712052	45,50
68	*S. epidermidis*	Wound secretion	6706013	93,10
69	*S. epidermidis*	Conjunctival secretion	6706011	93,20
89	*S. haemolyticus*	Wound secreton	6616051	53,30
-	*S. aureus* ATCC 25923	Reference strain	6336173	90,30

4.4. Oxacillin Minimum Inhibitory Concentration values

For Staphylococcus aureus oxacillin strains with MIC values equal and lower 2 mg/ml indicates their sensibility and strains with MIC values equal and higher than 4 mg/ml strains are considered resistant. In case of coagulase negative staphylococci the oxacillin MIC values are ≤0,25 µg/ml for oxacillin sensitive and ≥0,5 µg/ml for the oxacillin resistant strains [NCCLS, 2003].

Table 3 show the MIC values determinated using broth microdilutions method. Selected strains are confirmed to be meticilin-resistent by multiplex PCR method.

Table 3. MIC values and their interpretation for the 23 bacterial strains.

ID Nr.	Species	MIC for oxacillin (µg/ml)	Interpretation
1	*S. warneri*	256	R
8	*S. xylosus*	0,25	S
9	*S. haemolyticus*	256	R
15	*S. haemolyticus*	32	R
20	*S. xylosus*	16	R
25	*S. aureus*	16	R
26	*S. xylosus*	2	R
33	*Staphylococcus haemolyticus*	128	R

ID Nr.	Species	MIC for oxacillin (µg/ml)	Interpretation
47	*S. aureus*	128	R
51	*S. epidermidis*	64	R
54	*S. epidermidis*	64	R
55	*S. hominis*	16	R
66	*Staphylococcus aureus*	8	R
68	*S. epidermidis*	64	R
69	*S. epidermidis*	64	R
89	*S. haemolyticus*	32	R
96	*S. aureus*	256	R
99	*S. aureus*	256	R
106	*S. aureus*	64	R
107	*S. aureus*	128	R
111	*S. aureus*	8	R
116	*S. aureus*	128	R
117	*S. aureus*	128	R
-	*S. aureus* ATCC 25932	0,25	S

All bacterial strains but (strain of *Staphylococcus xylosus* identification number 8) one were confirmed to be resistant to oxacillin /methicillin. These results well correlates with those obtained by *mecA* gene amplification. The used control strain *Staphylococcus aureus* ATCC 25932 were confirmed sensitive to oxacillin which is in accord to the product specifications.

4.5. Amplification of regulatory genes of *mec* region (*mecI* and *mecR*)

MecR1 and *mecI* genes are involved in *mecA* gene regulation. In this case it is considered that the mutations occurring in these genes may have effects on the level of resistance to methicillin.

The *mecI* gene was amplified using $mecI_1$ and $mecI_2$ primers. Expected fragment of 416 bp was obtained for strains with ID number 9, 15, 20, 25, 55, 96, 99, 106, 107, 116 and 117 (Figure 7).

The *mecR1* gene was amplified using $mecR_1$ and $mecR_2$ primers. Expected fragment of 1922 bp was obtained for strains with ID number 9, 15, 20, 25, 96, 99, 106, 107, 116 and 117 (Figure 8).

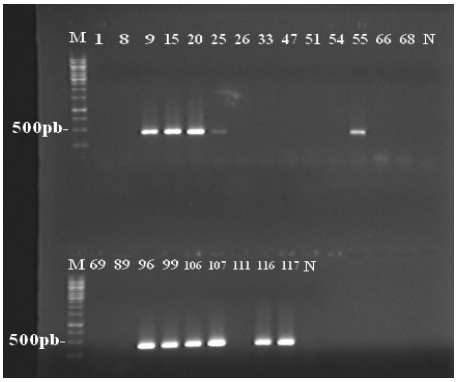

Figure 7. *MecI* gene amplification.

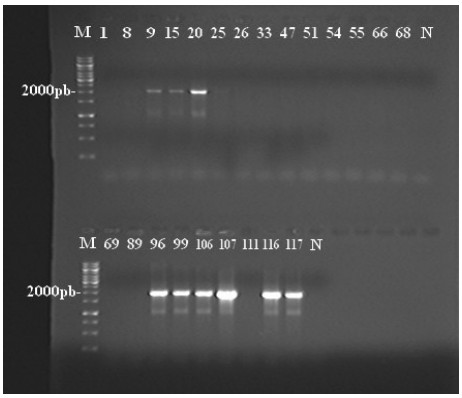

Figure 8. *MecR1* gene amplification.

The results of the two amplifications are in very close correlation. In case of strains with the complete configuration type A (1) *mec* gene complex, both specific fragments were amplified. Strains which show no amplification for any of the regulatory genes, have most likely type B(2), C(3) or D(4) *mec* complexes)[Suzuki et al, 1993]. An exception is the strain with ID number 55. This particular case is one for which no data was found in the literature, is present amplified fragment for *mecI* gene but not for *mecR1* gene. Perhaps this strain has emerged only mutation (deletion) of a *mecR1* gene fragment and it presents a new type of mec complex.

4.6. Sequence analysis of *mecI* gene

Sequencing reactions for all 11 samples were performed at Macrogen Inc. (Seoul, Korea). The data were processed using the program BioEdit Sequence Alignment Editor version 7.0.5.3.

After analyzing the received sequences we considered necessary to manually cut the first 20 nucleotides and analize datas until nucleotide 388 for all 11 samples. The sequences were grouped for alignment in two main categories: MRSA and CNS.

Sequences from *Staphylococcus aureus* strains were aligned with corresponding sequences of MRSA252, N315, Mu50 and Mu3 strains (Figure 9). We could notice point mutations present in Mu3 and Mu50 strains. It is a G-T transversions reported for position 43.

Figure 9. Gene sequences alignment of *Staphylococcus aureus* strains no. 25, 96, 99, 106, 107, 116 and 117 with the corresponding gene sequences of MRSA252, N315, Mu50 and Mu3 strains. Point mutations are highlighted in frames. NCBI identification numbers for the used strains are: MRSA252 -2861158, N315 -1122814, -5560220 Mu3, Mu50 -1120003.

This change affects only the primary structure of protein and not its function, because substitution in position 15 is between phenylalanine and valine; both are hydrophobic amino acids (figure 10).

For studied strains appears a transition; a cytosine is changed to thymine at position 202 (T 96, T 99, T 106, T 107, T 116 and T 117 strains). The structure of the resulting protein is changed, the synthesis beeing stopped at codon 68. The codon for glutamine (CAA) is substituted with a stop codon (TAA), being a well documented mutation [Suzuki et al., 1993, Kobayashi et al., 1998, Weller 1999; Klitgaard et al., 2008].

To highlight the implications of these mutations isoelectric point and molecular weight for both translated proteins were calculated. Thus, in case of MRSA252 strain pI/Mw values are 7,80 respectively 13680,67. In case of a shorter product, e.g. strain T 96, the pI/Mw values are 9.48 respectively 8145.51 [Gasteiger et al., 2005].

If we consider product whose synthesis is stopped suddenly inactive, *mecA* gene inhibition no longer occurs for strains in which this new mutation is present. The data are consistent with MIC values to oxacillin/methicillin determined for these strains, with values between 64-256 µg /ml.

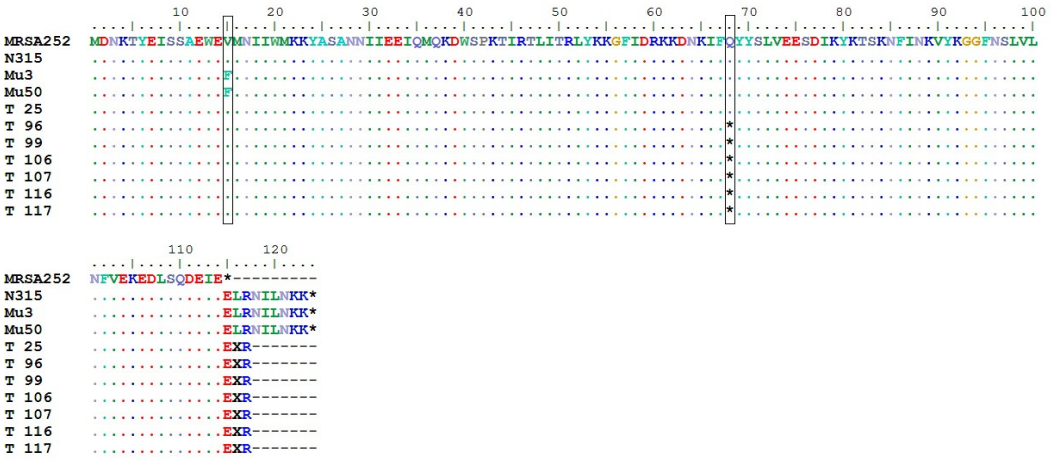

Figure 10. Alignment of translated *mecI* gene sequences of *Staphylococcus aureus* strains. Point mutations are highlighted in frames ; asterix is a stop codon. Access numbers of used protein sequences are: N315-BAB41258, MRSA252-CAG39070,-BAF76925 Mu3, Mu50-BAB56205.

In case coagulase negative strains there were no reported changes that may have phenotypic significance (Figure 11). These were analyzed in comparison with fully sequenced coagulase negative *Staphylococcus* strain RP62A.

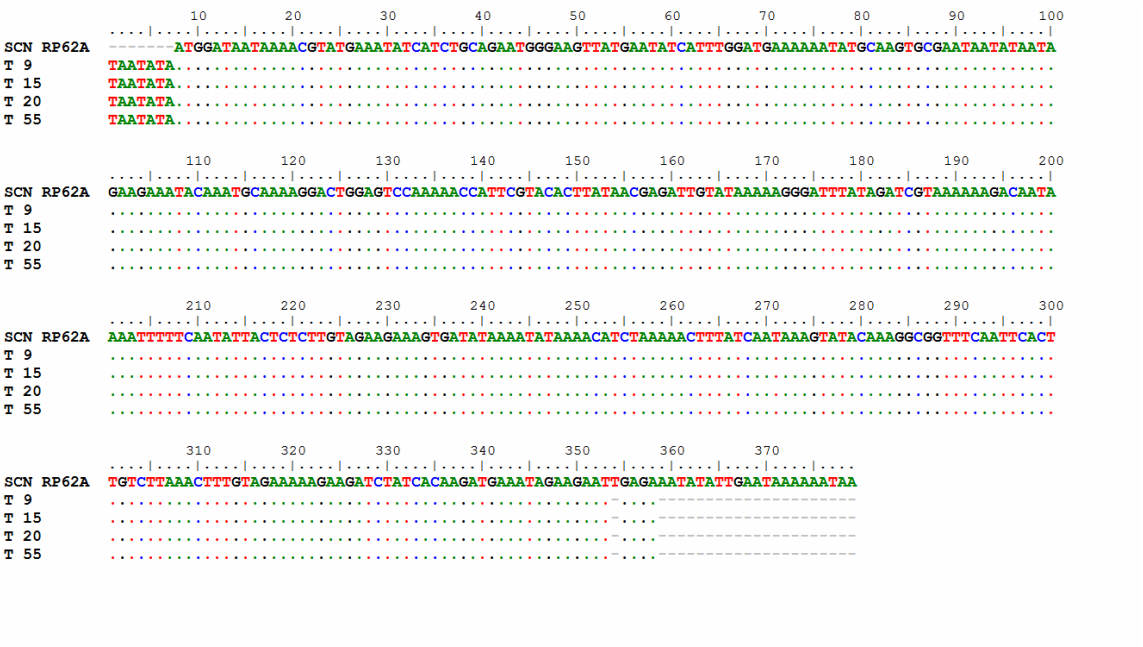

Figure 11. Alignment of *mecI* gene sequences of CNS strains. NCBI identification number for the strain used is 3242261.

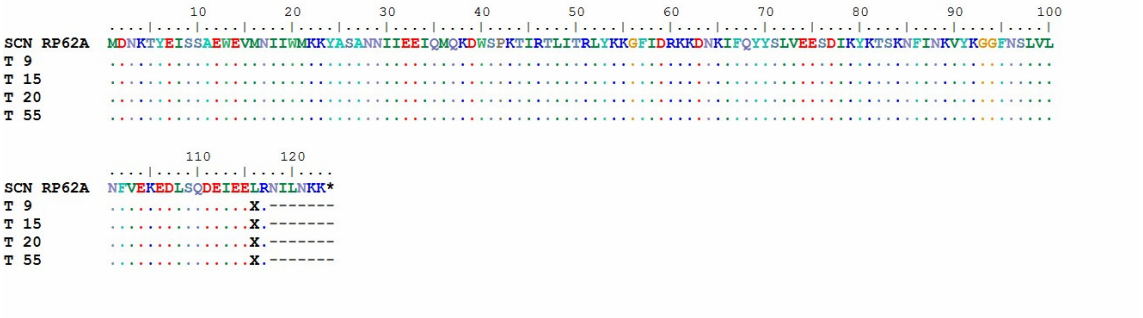

Figure 12. Alignment of the translated sequences of CNS strains. Acces number for used protein sequence is AAW53312.

The translated protein sequences are presented in Figure 12. The noted discrepancies at the beginning or at the end of sequence are most likely due to sequencing errors, by the presence of gaps or other nonconformities.

5. CONCLUSIONS

1. In this study there were evaluated diagnostic methods in infections caused by *Staphylococcus* genus.

We applied classical bacteriological methods, which involve bacterial culture, morphologic and stain character evaluation, biochemical tests and performing some sensibility testing by disc diffusion method.

Later the involved staphylococcal species were identified using API® Staph biochemical test system and a small number of strains were confirmed by automated VITEK® 2 Compact system.

Finally we tested the *mecA/ nucA* duplex PCR technique and sequence alalysis for *mecI* gene.

Currently the most used remains the traditional methods because they involves lower costs and thus generate valid results.

Molecular biology methods involves relatively high costs, well-trained staff, but they can also provide reproducible results in a short time. We consider therefore that in short time molecular methods will penetrated into the routine of microbiological diagnostic laboratories.

2. Using the classical microbiological methods we could successfully identify strains of *Staphylococcus aureus* and CNS.

3. Duplex PCR method *mecA /nucA* a was applied to the 125 strains, and 21.60% of them were *mecA* positive; 38.40% of all tested strains were positive for the *nucA* gene and they were thus cosidered *Staphylococcus aureus* strains .

4. Identifications made using API Staph biochemical tests system revealed a number of 4 strains of *Staphylococcus epidermisis*, 4 strains of *Staphylococcus haemolyticus*, three strains of *Staphylococcus xylosus*, one strain of *Staphylococcus hominis* and a strain of *Staphylococcus warneri*.

5. Minimum inhibitory concentrations for selected strains were ranged between 0.25 and 256 mg /ml oxacillin.

With one exception, strain 8 *Staphylococcus xylosus,* all bacterial strains were confirmed to be resistant to oxacillin /methicillin, results that correlate with those obtained by *mecA* gene amplification method.

6. Amplification of *mec* region regulatory genes revealed 11 *mecI* positive strains and 10 *mecR1* positive strains.

The results of the two amplifications are closely interrelated.

Exception it is number 55 strain, where it was noticed the presence of an amplification product for *mecI* gene but not for *mecR1*gene. Perhaps this strain has a particular type of *mec* complex; no similar data were found in the literature.

7. *MecI* gene sequencing revealed a single point mutation in case of the following *Staphylococcus aureus* strains: 96, 99, 106, 107, 116 and 117. Cytosine-thymine transition was observed at position 202.

The structure of the protein is modified, the synthesis beeing stopped at position 68. Glutamine in this position (CAA) is substituted with a stop codon (TAA). Short-chain MecI protein will be no longer functional and *mecA* gene will be consitutively translated.

The data are in close correlation with the oxacillin MIC values determined for these strains.

8. Further investigations are needed to elucidate the genetic mechanisms of heterogeneous methicillin resistance.

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