RAPID IDENTIFICATION OF METHICILLIN-RESISTANT STRAINS OF STAPHYLOCOCCUS GENUS USING MOLECULAR TECHNIQUES

M. COLCIERU[1], E. JAKAB[2], O. POPESCU[3]

[1] C.F. General Hospital Sibiu, [2,3]Universitatea "Babeş-Bolyai", Cluj-Napoca

Keywords: *MRSA – methicillin-resistant Staphylococcus aureus, MRSS- methicillin-resistant Staphylococcus strains, MSSA- methicillin-susceptible Staphylococcus aureus, MSSS - methicillin-susceptible Staphylococcus strains, PCR-polymerase chain reaction, strain, gene.*

Abstract: *Nosocomial infections cause serious problems in most countries. Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most incriminated germs. Of course there are traditional methods for determining susceptibility to methicillin, but the phenotypic expression of this resistance often shows a heterogeneous nature. (1) We tested the methicillin resistance and confirmed the strain using mecA/nucA duplex PCR technique (2). The mecA gene encodes the penicillin binding protein 2a (PBP2a) that is characteristic to methicillin-resistant staphylococci (3). The nucA gene encodes a thermostable nuclease. The amplified fragment is unique to the S. aureus strains (4). The size of the amplified product was 533 bp in the case of the mecA gene, respectively 279 bp for the nucA gene. The amplified products were separated by agarose gel electrophoresis. A total of 93 staphylococcus strains were tested using the mecA/nucA duplex PCR technique. The genotypic duplex PCR method lasts maximum 18 hours.*

Cuvinte cheie: *MRSA – methicillin - resistant Staphylococcus aureus, MRSS - methicillin-resistant Staphylococcus strains, MSSA - methicillin-susceptible Staphylococcus aureus, MSSS - methicillin-susceptible Staphylococcus strains, PCR - polymerase chain reaction, tulpină, genă*

Rezumat: *Infecţiile nozocomiale sunt o problemă serioasă în cele mai multe ţări. Staphylococcus aureus rezistent la meticilină (MRSA) reprezintă unul din cei mai incriminaţi germeni. Bineînţeles există metode clasice pentru determinarea susceptibilităţii la meticilină dar expresia fenotipică a acestei rezistenţe deseori reprezintă un caracter heterogen. (1) S-a efectuat un test de determinare simultană a rezistenţei la meticilină şi confirmarea speciei cu ajutorul tehnicii duplex PCR mecA/nucA (2). Gena mecA codifică proteina de legare a penicilinei 2a (PBP2a) care este caracteristică pentru stafilococii rezistenţi la meticilină (3). Gena nucA codifică nucleaza termostabilă de la Staphylococcus aureus, fragmentul amplificat fiind caracteristic tulpinilor de S. aureus (4). Amorsele oligonucleotidice folosite pentru amplificarea fragmentelor generează în final pentru gena mecA un produs de reacţie de 533 pb, iar pentru gena nucA un produs de aproximativ 279 pb, vizualizaţi cu ajutorul electroforezei în gel de agaroză. Tehnica duplex PCR mecA/nucA a fost aplicată pe un lot de 93 de tulpini de stafilococ. Această metodă permite identificarea tulpinilor MRSA în aproximativ 18 ore.*

INTRODUCTION

Nosocomial infections cause serious problems in most countries. Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most incriminated germs. Of course there are traditional methods for determining susceptibility to methicillin, but the phenotypic expression of this resistance often shows a heterogeneous nature.

PURPOSE OF THE STUDY

The purpose of the present study is to demonstrate the possibility of early identification of methicillin-resistant staphylococci through the mecA/nucA duplex PCR technique.

MATERIAL AND METHOD

Bacterial strains and growing conditions

Bacterial strains were isolated in Sibiu between 16.07.2008 and 10.07.2009. They were identified by using conventional microbiological methods: bacterial culture, biochemical tests and antibiotic-sensitivity tests. Pathological samples were diverse: pus, otic, conjunctival, nasal, and urethral secretions, sputum etc.

Oligonucleotide samples

Oligonucleotide primers used for PCR reaction in the case of the *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 (3).

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 bound to the 447 base pair long *nucA* gene which encodes the A thermonuclease. The primer 1 bound to 49 and 69 nucleotide positions, and the primer 2 bound to 304 and 327 nucleotide positions. Thus a 279 pb long fragment was generated (4). The oligonucleotide primers were supplied by the Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary.

Bacterial sample preparation

A bacterial suspension made by suspending a colony in 15µl of pure water was used for direct determination. Based on the results of the duplex PCR the methicillin-resistant strains were selected, the bacterial DNA was isolated and re-tested by PCR. Lysis buffer contains 0.2 mg/ml lizostaphin, 20 mM Tris/HCl, 2 mM EDTA, 1% Triton X-100 at pH 8 (5). Later, proteinase K were added and the DNA was purified using Nucleospin® Tissue kit (Macherey-Nagel, Düren, Germany).

PCR technique

PCR mix contains: 5x concentrated Green reaction buffer,

[1]Corresponding Author: M. Colcieru, C.F.General Hospital, Sibiu, 20 C-tin Noica street, Sibiu, Romania, e-mail:_mcolcieru@yahoo.com @yahoo.com, tel +40-0723 809490

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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 minute 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 minute at 72°C. Steps 2-4 were 30-fold repeated (6).

Agarose gel electrophoresis of the amplicons

For the separation of the duplex PCR 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, Lituania) or 1 kb DNA Ladder (Axigen Biosciences, Union City, SUA).

RESULTS

Duplex PCR technique was used to test 93 strains. 21.5% of them were *mecA* positive; 33.33% of all tested strains were positive for the gene *nucA* so they were confirmed as *Staphylococcus aureus.* The remnants of the *nucA* negative isolates belongs to other *Staphylococcus* species (Table 1).

Table no. 1. Number of strains and their share according to the presence or absence of *mecA / nucA* gene.

nr.	FEATURE	NR. OF STRAINS	SHARE %
1	$mecA^+$	20	21,50
2	$mecA^-$	73	78,50
3	$nucA^+$	31	33,33
4	$nucA^-$	62	66,66
5	$mecA^+nucA^+$	6(MRSA)	6,45
6	$mecA^+nucA^-$	14(MRSS)	15,05
7	$mecA^-nucA^+$	25(MSSA)	26,88
8	$nucA^-nucA^-$	48(MSSS)	51,61

Only the methicillin-resistant strains were selected (1, 8, 9, 15, 20, 25, 26, 27, 33, 47, 51, 54, 55, 60, 66.68, 69, 89, 92), the bacterial DNA was isolated, purified and re-tested with the same PCR method. However strains 73, 75, 77, likely to be MRSA, were lost during the first passage.

Calculation of DNA concentration

In the present study we used a NanoDrop ND-1000 spectrophotometer to analyse 19 samples. The absorbance ratios and DNA concentration expressed in ng/µl have been summarized in Table 2. After isolation and purification of the genomic DNA, we proceeded to PCR amplification under the same conditions (Figure 1 and 2).

The strain 47 is MRSA. Amplification products are present in both cases of the *mecA* gene (533 bp) and the *nucA* gene (279 bp). Another strain of interest is strain 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 is not as intense as in the case of the strain 47.

Strain 25 shows a heterogeneous methicillin-resistance. Strain 66 is also 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.

Table no. 2. Measurement of DNA concentration with NanoDrop ND-1000.

Nr.	SAMPLE	260NM/280NM	260NM/230NM	NG/ML
1	1	2,02	1,93	45,6
2	8	1,91	2,21	98,3
3	9	1,94	1,98	90,6
4	15	1,87	1,97	97
5	20	2,06	2,36	101,2
6	25	1,98	2,36	109,6
7	26	1,98	1,88	68,8
8	27	2,19	2,47	797
9	33	1,94	2,49	290,8
10	47	2,04	2,43	350
11	51	2,11	2,17	245,5
12	54	2,05	2,37	200,8
13	55	2,14	2,37	258,5
14	60	2,1	2,41	558,6
15	66	2,02	2,29	108,8
16	68	2,06	2,36	162,6
17	69	2,18	2,3	178,3
18	89	2,01	2,14	42,8
19	92	2,08	2,25	72,5

Figure no. 1 Agarose gel electrophoresis of amplification products for strains 1, 8, 9, 15, 20, 25, 26, 27, 33, and 47.

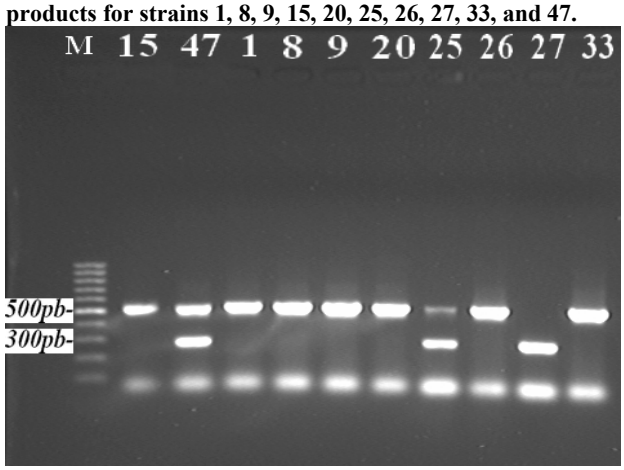
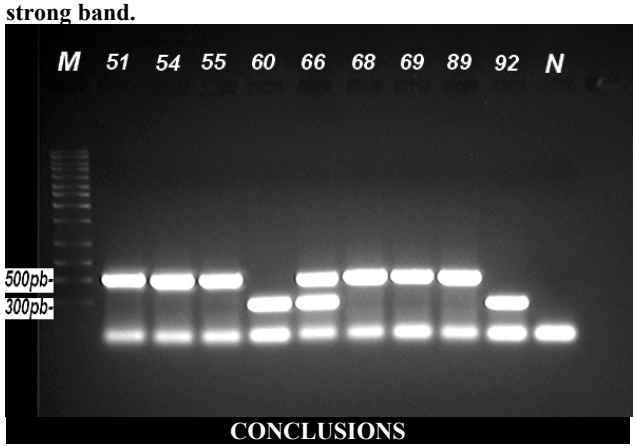


Figure no. 2 Agarose gel electrophoresis of amplification products after DNA isolation for strains 51, 54, 55, 60, 66, 68, 69, 89, and 92; on the last position you can see the presence of negative control (N); the unused primers form a strong band.



CONCLUSIONS

Early identification of methicillin-resistant strains allows the implementation of prevention and control strategies

to reduce total costs of *Staphylococcus* infections.

The standard procedures of clinical microbiology encounter difficulties most frequently in the identification of MRSA strains, especially when the resistance has a heterogeneous character. The conventional techniques give results in 2-3 days.

Duplex PCR *mecA/nucA* method allows us a rapid identification of methicillin-resistant strains, particularly MRSA strains, in maximum 18 hours.

The specificity of primers used for PCR technique demonstrates that the presented method is quite sensitive and it can be applied for MRSA detection.

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