



# Molecular-genetic Method for Fast Direct Detection of *Staphylococcus Aureus* and Methicillin Resistance in Blood Cultures and Punctures

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## Abstract

**Background:** Invasive infections caused by methicillin resistant *Staphylococcus aureus* and coagulase-negative staphylococci (MRSA/MRSCoN) require fast, adequate treatment.

**The aim** of this study was to develop a faster protocol for direct detection of MRSA/MRSCoN in blood cultures and in abscess punctures based on *mecA* and species specific identification of *S. aureus* by polymerase-chain reaction (PCR).

**Materials and methods:** We examined 77 growth-positive BACTEC blood cultures and 50 abscess punctures by routine microbiological assay and simultaneous PCR detection of MRSA/MRSCoN. The specificity of the PCR was evaluated by using DNA from another 15 microbial species for negative controls. We determined the minimum inhibitory concentration (MIC) of oxacillin, vancomycin, tigecycline, linezolid, levofloxacin, clindamycin, and erythromycin against the *S. aureus* isolates using the E-test.

**Results:** In the blood cultures, the two methods detected 39.3% of MRSA, and 93.9% of MRSCoNS. In the punctures, the PCR assay identified 20.9% of MRSA and 79.2% of MRSCoNS. In the puncture cases, there were three PCR MRSA positive and culture negative samples. Screening for susceptibility to 14 antimicrobial agents demonstrated significantly higher ( $p < 0.05$ ) methicillin resistance in blood culture isolates than in the puncture ones (39.3% and 20.0%, respectively).

**Conclusion:** The new PCR protocol was very fast and specific. It was more sensitive in detecting MRSA from abscess punctures than the routine microbiological techniques. This protocol will speed up the right choice of empirical therapy, which is extremely important for saving patients' lives.

## Keywords

blood cultures, methicillin resistance, punctures, PCR, *Staphylococcus aureus*,

## INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase-negative staphylococci (MRSCoNS), except when the latter are contaminants, are the major causes of life-threatening bloodstream infections

(BSI), endocarditis, postoperative soft tissue infections, osteomyelitis, septic arthritis, metastatic abscesses, especially nosocomial ones, and pose a global problem for public health.<sup>1-3</sup> The virulence arsenal of staphylococcal isolates differs depending on the bacterial species and the coagulase-positive *S. aureus* has been proved to be the most virulent species. The most frequent severe BSI infections caused

by this pathogen are due to various extracellular products, e.g. enzymes, hemolysins and other toxins that play a role as virulence factors.<sup>4-6</sup> *S. aureus* has been also detected as one of the common etiological agents of respiratory infections, especially in Bulgarian children over the period from 1998 to 2014.<sup>7</sup> This microorganism has been the one most often isolated from Bulgarian patients with lesions due to chronic venous disease, peripheral arterial occlusive disease of the lower extremities, and advanced diabetic microangiopathy.<sup>8</sup>

The typical diagnostic algorithm for invasive staphylococcal infections is isolation of pure microbial culture and its identification and detection of antimicrobial susceptibility that requires at least three days to perform by conventional techniques.<sup>9</sup> The polymerase chain reaction (PCR) is very fast and specific approach, an alternative to routine cultural method.<sup>9,10</sup>

The *mecA* gene is the major determinant of methicillin resistance or phenotypic expression of heteroresistance. This chromosomal genetic element appears in both MRSA and MRCoNS with production of low-affinity penicillin-binding protein, and consequently, resulting in cross-resistance to most  $\beta$ -lactam antimicrobials and the significant limitation in their use.<sup>10,11</sup> It is possible to identify *S. aureus* and/or detect the presence of *mecA* using polymerase-chain reaction (PCR) assay in isolated staphylococcal strains or directly in blood cultures or in animal samples.<sup>10,12</sup>

The aim of this study was to develop a new algorithm for rapid detection of MRSA/MRCoN and methicillin sensitive *S. aureus* (MSSA) directly in growth-positive blood cultures and/or in abscess punctures by *mecA* detection and species-specific identification of *S. aureus* by PCR and to recommend adequate therapy based on the susceptibility of the MRSA isolates.

## MATERIALS AND METHODS

### SAMPLES, STRAINS AND ANTIMICROBIAL SUSCEPTIBILITIES

BACTEC blood cultures flacons (BBL, Germany) (N=77) inoculated with blood samples from patients after signalled as growth-positives and punctures (N=50) from abscesses before cultivation were subjected to microscopic examination. PCR testing was done after the finding of Gram-positive cocci in clusters from microscopically examined samples. We performed simultaneous routine microbiological examination for all samples.

Isolation and presumptive identification of pure culture Gram-positive cocci with grape-like cluster arrangement were done under routine criteria.<sup>9</sup> We used Crystal GP (BBL, Germany) for more detailed biochemical species identification.

The antimicrobial susceptibility assay was performed by the disc diffusion method for benzylpenicillin (1 unit), cefoxitin (30  $\mu$ g), erythromycin (15  $\mu$ g), clindamycin (2  $\mu$ g), gen-

tamicin (10  $\mu$ g), chloramphenicol (30  $\mu$ g), trimethoprim/sulphamethoxazole (25  $\mu$ g), levofloxacin (5  $\mu$ g), tigecycline (15  $\mu$ g), and vancomycin (5  $\mu$ g). The antibiotic discs were purchased from HiMedia, Laboratories Pvt. Limited, Mumbai, India. Each isolate was studied and evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines<sup>13</sup> (<http://www.euca.org>). *S. aureus* ATCC 25293 (MSSA) and ATCC 43300 (MRSA) were used as quality control strains.

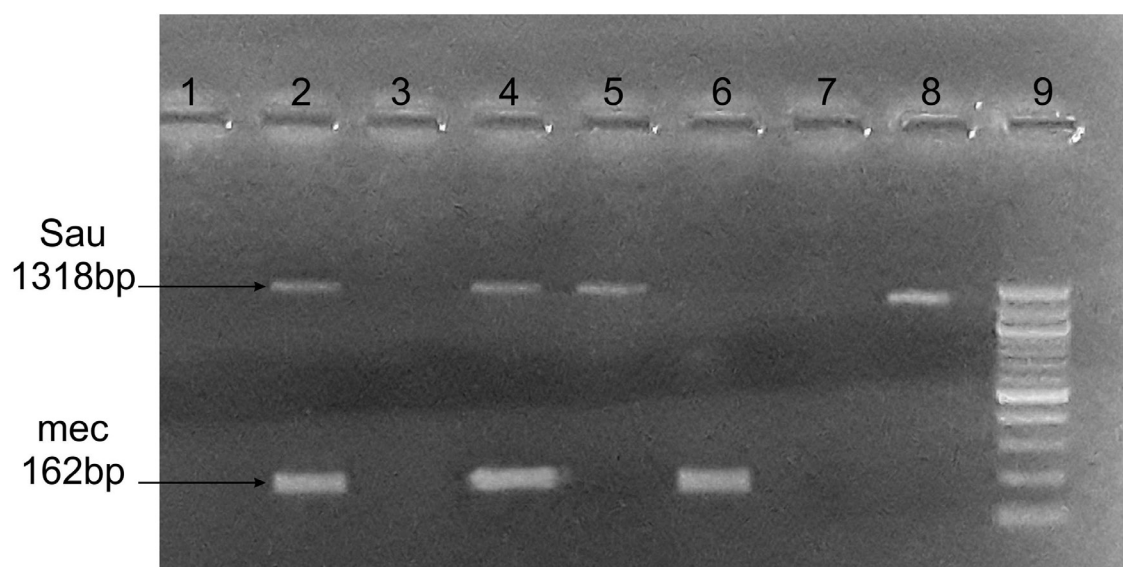
Minimum inhibitory concentration (MIC) of oxacillin, vancomycin, tigecycline, linezolid, levofloxacin, clindamycin and erythromycin against *S. aureus* isolates was determined with the E-test (Laboratories Pvt. Limited, Mumbai, India). Suspension from pure staphylococcal culture standardized to 0.5 McFarland was inoculated on a Muller-Hinton agar (MHA) plate purchased from HiMedia, Laboratories Pvt. Limited, Mumbai, India. An E-test strip was placed and then the sample was incubated for 18 $\pm$ 2 h at 36°C. The results were evaluated according to EUCAST 2018 (<http://www.euca.org>).

### DNA EXTRACTION AND POLYMERASE CHAIN REACTION (PCR) ASSAY

Total genomic DNA was extracted from the blood cultures and punctures (127 samples) using a DNA sorb-AM nucleic acid extraction kit (AmpliSens, InterLabService, Moscow, Russia), in accordance with the manufacturer's instructions.

PCR was performed in a 25  $\mu$ l reaction mix. *S. aureus* were genetically identified by PCR assay with specific primers for this bacterial species – *Sau327* 5'-GGACGACATTAGACGAATCA-3' and *Sau 1645* 5'-CGGGCACCTATTTTC-TATCT-3' 23S rRNA with an amplicon size of 1318 bp (**Fig. 1**).<sup>6</sup> Methicillin resistance was determined by detection of *mecA* with the following primer sequences: F5'-TCC AGA TTA CAA CTT CAC CAG G-3' and R 5'-CCA CTT CAT ATC TTG TAA CG-3' with an amplicon size of 162 bp (**Fig. 1**).<sup>1</sup> The primer sequences were verified for specificity using the Basic Local Alignment Search Tool (BLAST) program available from the NCBI website (Bethesda, MD) (<http://www.ncbi.nlm.nih.gov/BLAST>). DNA was amplified after optimisation of the PCR protocol: initial denaturation at 94°C for 5 min and 35 cycles of denaturation at 94°C for 2 min; annealing at 57°C for 2 min; extension at 72°C for 1 min; final elongation at 72°C for 7 min.

The specificity of the PCR assay was evaluated by using genome DNA from another 15 microbial species isolated directly from blood cultures and punctures, predominantly Gram-positive ones: *Streptococcus pneumoniae*, *S. pyogenes*, *S. agalactiae*, *S. bovinum*, *S. sanguis*, *Enterococcus faecalis*, *E. faecium*, *Listeria monocytogenes*, *Corynebacterium pseudodiphtheriticum*; and some Gram-negative ones: *Escherichia coli*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*; and two species of fungi *Candida albicans* and *C. parapsilosis*.



**Figure 1.** Multiplex PCR detection of *S. aureus* and *mecA* for methicillin resistance.

Electrophoretic separation of amplicons presented in bp (*Sau*1318bp and *mecA*162bp). 1 – negative control with *E. faecalis* in blood culture; 2 – positive sample MRSA in blood culture; 3 – negative control with *E. coli* in blood culture; 4 – positive control with ATCC 43300 MRSA; 5 – positive sample MSSA in blood culture; 6 – positive sample only for *mecA* (MRSCoN) - *S. epidermidis* in blood culture; 7 – negative control with *S. pyogenes* in puncture; 8 – positive sample MSSA in puncture; 9 – DNA ladder.

#### STATISTICAL ANALYSIS

Descriptive statistics, chi-square test and Fisher's exact test were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, United States). Results were considered statistically significant at  $p < 0.05$ .

#### RESULTS

In this study, we obtained 77 growth-positive blood cultures with Gram-positive cocci in clusters determined based on both of the methods used (PCR assay and microbial culture). Of these, 36.4% were *S. aureus*, and 39.3% - MRSA.

**Table 1.** Comparison of results for detection of methicillin resistance by using PCR and routine testing

Group I. Staphylococcal isolates from 77 growth positive blood cultures	Identification by PCR	Identification by routine method	<i>p</i> -value*	Methicillin resistance ( <i>mecA</i> ) detected by PCR	Methicillin resistance detected by Cefoxitin disc	<i>p</i> -value*
<i>S. aureus</i>	28	28	1.0000	11	11	1.0000
<i>S. epidermidis</i>		42		39	39	1.0000
<i>S. haemolyticus</i>		2		2	2	1.0000
<i>S. capitis</i>		1		1	1	1.0000
<i>S. cohnii</i>		2		2	2	1.0000
<i>S. lugdunensis</i>		1		1	1	1.0000
<i>S. warneri</i>		1		1	1	1.0000
Group II. Staphylococcal isolates from 50 abscess puncture	Identification by PCR	Identification by routine method	<i>p</i> -value*	Methicillin resistance ( <i>mecA</i> ) detected by PCR	Methicillin resistance detected by Cefoxitin disc	<i>p</i> -value*
<i>S. aureus</i>	48	45	0.2421	10	7	0.2105
<i>S. lugdunensis</i>		1				
<i>S. epidermidis</i>		1		1	1	

\* Statistically significant at  $p < 0.05$ .

Overall, 63.6% were coagulase negative staphylococci (CoNS), and 93.9% of them were MRCoNS (Table 1). Out of 50 punctures, we isolated 15.6% MRSA (using PCR these were determined to be 20.8% of MRSA (Table 1). Three puncture samples did not produce any microbial growth, but were positive for MRSA by PCR. Some of the results are presented in Fig. 1.

All of the blood culture samples with different bacterial species isolates (*Streptococcus pneumoniae*, *S. agalactiae*, *S. bovinum*, *S. sanguis*, *Enterococcus faecalis*, *E. faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*, *Candida albicans* and *C. parapsilosis*, and a *S. pyogenes* isolate from puncture lacked amplicons for *Sau* and *mecA* genes in the PCR assay. A few negative controls were used in the electrophoresis (Fig. 1).

Next, we screened the isolates for susceptibility to 14 antimicrobial agents. The results showed significantly higher ( $p=0.01$ ) methicillin resistance (39.3%) among the blood culture isolates (group I) than among the ones from abscess (group II) - 15.6%. The PCR and the routine microbiological results for the blood samples (Group I) were fully consistent. The MIC value of oxacillin was in the range of 4

-  $\geq 256$  mg/l. MRSCoN were up to 93.9% of all the CoNS isolates from blood cultures, again determined by both methods. The resistance to benzylpenicillin showed similar prevalence, about 90%, in both of the groups (Table 2). The resistance to more than 3 groups of antimicrobials (i.e. multidrug resistance, MDR) was at 20.0% among the isolates from group I and in 15.6% among ones from group II. The isolates from group I showed no significant prevalence of MDR compared to the tested ones from group II ( $p=0.54$ ). MLS (macrolides, lincosamides, streptogramins) resistance was no significantly different (Table 2) between the isolates from abscess punctures (37.8% to macrolides and 24.4% to lincosamides) and the ones from blood cultures (respectively 32.0% and 20.0%). No staphylococcal isolate resistant to vancomycin, tigecycline and linezolid was detected (Table 2). There was just one MRSA with co-resistance to minocycline (MIC 8 mg/l).

## DISCUSSION

The presence of MSSA and *mecA* gene, resp. MRSA and MRCoNS in 127 patient's samples from blood cultures and

**Table 2.** Resistance to various groups antimicrobials

Antibiotic	<i>S. aureus</i> isolates from Group I. blood culture resistant - N/ total - N (%) (MIC range mg/l)	<i>S. aureus</i> isolates from Group II. abscess resistant - N/ total - N (%) (MIC range mg/l)	<i>p</i> value*
Benzylpenicillin	22/28 (89.29)	41/45 (91.11)	0.1679
Cefoxitin	11/28 (39.29)	7/45 (15.56)	0.0100 *
Oxacillin	(4 - $\geq 256$ )	(4 - 32)	
Erythromycin	8/28 (28.57) (0.125 - $\geq 256$ )	17/45 (37.78) (0.25 - $\geq 256$ )	0.4583
Clindamycin	5/28 (17.86) (0.125 - $\geq 256$ )	11/45 (24.44) (0.125 - $\geq 256$ )	0.5730
Gentamicin	3/28 (10.71)	1/45 (2.22)	0.1543
Chloramphenicol	0/28 (0)	1/45 (2.22)	1.0000
Tetracyclin	4/28 (14.29)	2/45 (4.44)	0.1951
Minocycline	0/28 (0) (0.125 - 0.5)	1/45 (2.22) (0.125 - 8)	1.0000
Trimeth./sulph.**	3/28 (10.71)	3/45 (6.67)	0.6692
Levofloxacin	0/28 (0) (0.125 - 0.5)	1/45 (2.22) (0.125 - 8)	1.0000
Vancomycin	0/28 (0) (1 - 2)	0/45 (0) (1 - 2)	1.0000
Linezolid	0/28 (0) (1 - 2)	0/45 (0) (1 - 4)	1.0000
Tigecycline	0/28 (0) (0.064 - 0.25)	0/45 (0) (0.064 - 0.25)	1.0000

\* statistically significant ( $p < 0.05$ ).

\*\* Trimethoprim/sulphamethoxazole

abscesses was detected simultaneously by PCR assay and routine microbial cultivation. The PCR only took a few hours in contrast to routine technique. The testing of 15 different gram-positive, gram-negative and fungal isolates from blood cultures and punctures was demonstrated absolutely specific negative results. The PCR assay was more sensitive in detecting MRSA from abscess punctures in this study than routine microbiological examination, a finding which needs further confirmation. An abscess sample, similar to other biological fluids, consists of many factors of the host such as inflammatory mediators, the complement system, phagocytic cells with bactericidal effect and when the living bacterial cells are not enough or do not grow in vitro, it can detect only bacterial DNA.<sup>14</sup> The PCR assay was more sensitive in detecting MRSA from biological fluids, especially when the living bacterial cells are not enough or do not grow in vitro.

In the present study, bacteriemia was due to strong virulent *S. aureus* in more than 36% of the cases caused by gram-positive bacteria. The abscesses with the same etiology were in 96% of the cases, which is a very important reason to immediately perform exact diagnosis by PCR. Our results confirm that the major opportunistic etiological agent of nosocomial bacteriemia is a part of the indigenous microbiota of human skin and mucous membranes, *S. epidermidis*.<sup>15-17</sup> In this study, it was isolated in 54.6%, and 50.7% were MRSE from the tested blood cultures. Reportedly, 30.6% of all the cases of hospital acquired bacteriemia in Bulgaria between 2011 and 2016 were due to *S. epidermidis*.<sup>17</sup> Other CoNS (Table 1) were very rare isolates in the studied samples, similar to other results.<sup>15-17</sup> The significance of *Staphylococcus* spp. as a cause of health care-associated infections, especially BSI, has recently been a subject of research. Risk factors for infection due to CoNS are implanted medical devices, such as heart valve prostheses, orthopedic endoprostheses or peripheral and central venous catheters, commonly used in hospitals or immune-suppression in patients. *S. epidermidis* and *S. hemolyticus* produce strong extracellular slime and protein-binding substances that stimulate easy biofilm formation.<sup>17-19</sup> *S. warneri* are isolated rarely from patients with artificial heart valves, cerebrospinal fluid derivation shunts, intravascular catheters, similar to the other representatives of CoNS, but some cases of sepsis in immune-competent patients with lack of risk factors have been reported.<sup>2</sup> The first case of *S. warneri* isolated from sturgeons with sepsis has been reported by Bulgarian authors.<sup>20</sup> Another rare pathogen that can be isolated from patients with risk factors is *S. capitis*. There are some reports about its role in peritoneal dialysis peritonitis, pacemaker and prosthetic-valve endocarditis, meningitis, osteomyelitis and others.<sup>3,21</sup> The clone *S. cohnii* subsp. *cohnii* hu-01 is isolated from hospital environments with increasing frequency compared to other CoNS in China and Poland in recent years.<sup>22,23</sup> *S. lugdunensis*, which was isolated in only 2% of the examined patients' abscess punctures and in 1.3% of the blood cultures, can cause clinically significant infections, especially skin and soft tissue

ones, prevalent in older individuals, but bacteriemia, septic arthritis, toxic shock syndrome and postoperative endophthalmitis have been observed with this etiology. Its virulence is higher than that of other CoNS and this species has common virulence factors with *S. aureus*. As of now, it can be described as a rare potentially life-threatening pathogen. Most *S. lugdunensis* isolates originate from abscesses, cellulitis, osteomyelitis, or other wounds.<sup>24</sup>

The increasing number of hospital-acquired infections, especially BSI due to *Staphylococcus* spp., in recent years is closely linked to the spread of the *mecA* gene in strains living in hospital environment, which facilitates their ability to survive there.<sup>4,6</sup> In the present study, *mecA* was detected in 77.9% of the growth-positive blood cultures with staphylococcal isolates. Accordingly, bacteriemia with staphylococcal etiology is complicated by the emergence of methicillin-resistant pathogens. This is another reason to use the fast PCR assay for accurate detection of *mecA* in both MRSA and MRSCoNS invasive isolates. This method is very useful for the right choice of etiological therapy, which is extremely important for eradication of resistant causative agents and saving the patients' lives.

Often methicillin resistance occurs in combination with resistance to other various antimicrobials.<sup>22,23</sup> After susceptibility testing to other antibiotic groups, in this study we found that the MRSA isolates from blood cultures (at 39.2%) were predominated compared to those from abscesses (15.6%) ( $p=0.01$ ). The isolates from group I were from inpatients with bacteriemia. Nearly half of the cases ( $N=21$ ) were outpatients and 29 were inpatients. This suggests that there is a difference in the origin of the causative agent – community acquired or hospital acquired, which is more frequently described as a reason for increasing the health care-associated infections in relation to MRSA and MRSCoNS.<sup>6,17,22</sup> MLS resistance that did not correlate with hospital acquired infections. Moreover, a dramatic increase in clindamycin resistance among *S. aureus* isolates has been described.<sup>25,26</sup> The most frequent co-resistance to methicillin, MLS, tetracycline, gentamicin, trimethoprim/sulphamethoxazole was determined in the presently examined *S. aureus* isolates. New data about co-resistance to methicillin, vancomycin, tigecycline and linezolid has been described in India in 2013 – 2015,<sup>26</sup> but in Bulgarian invasive isolates (between 2016 and 2018), there has not been detected co-resistance to methicillin and these antimicrobials that can be used for empirical therapy after the establishing methicillin resistance with PCR.

## CONCLUSIONS

A fast algorithm for detection of *S. aureus* and *mecA* in growth-blood cultures and direct in abscess punctures by multiplex PCR assay and simultaneous routine microbial culture is reported. This new method is rapid, specific and more sensitive for determining MRSA from punctures than the routine microbiological technique. The growing expan-

sion of MRSA, and MRSCoNS causing bacteriemia and the major role of *S. aureus* in the etiology of abscesses makes it necessary to use PCR for immediate diagnosis of staphylococcal etiology and methicillin resistance. The data from additional examination of the susceptibility of *S. aureus* isolates to various antimicrobials can be used for the recommendation of the adequate therapy for severe invasive infections with MRSA or MSSA etiology. This algorithm speeds up the right choice of empirical therapy, which is extremely important for saving patients' lives.

## ACKNOWLEDGMENTS

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## ETHICS STATEMENT

There was no personal patient information in the database. The empirical therapy prescribed was as all the clinical practice and guidelines approved, according the current pathogen.

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# Молекулярно-генетический метод для быстрой прямой идентификации *Staphylococcus Aureus* и устойчивости к метициллину в посевах и пункциях крови

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## Абстракт

**Введение:** Инвазивные инфекции, вызванные устойчивым к метициллину *Staphylococcus aureus* и коагулазонегативными стафилококками (MRSA / MRSCoN), требуют быстрого и адекватного лечения.

**Цель** настоящего исследования состояла в том, чтобы разработать более быстрый протокол для непосредственного обнаружения присутствия MRSA / MRSCoN в культурах крови и при пункциях абсцесса на основе mecA и видоспецифической идентификации *S. aureus* с помощью полимеразной цепной реакции (PCR).

**Материал и методы:** Мы изучили 77 положительных ВАСТЕС гемокультур и 50 пункций абсцесса с помощью обычных микробиологических анализов и одновременной PCR -идентификации MRSA / MRSCoN. Специфичность PCR оценивали с использованием ДНК из ещё 15 видов микроорганизмов для отрицательного контроля. Мы определили минимальную ингибирующую концентрацию (МИК) oxacillin, vancomycin, tigecycline, linezolid, levofloxacin, clindamycin и erythromycin в отношении изолятов *S. aureus* с помощью E-test.

**Результаты:** В культурах крови оба метода идентифицировали 39,3% MRSA и 93,9% MRSCoNS. При пункционном PCR -анализе выявлено 20,9% MRSA и 79,2% MSSA. В случаях пункции было три положительных и отрицательных образца PCR MRSA. Скрининг чувствительности 14 противомикробных препаратов показал значительно более высокую ( $p < 0,05$ ) устойчивость к метициллину у изолятов культуры крови, чем у пункций (39,3% и 20,0% соответственно).

**Выводы:** Новый протокол PCR оказался очень быстрым и точным. Он оказался более чувствителен в определении MRSA пункций абсцесса, чем обычные микробиологические методы. Этот протокол поможет быстро установить правильный выбор эмпирической терапии, которая является чрезвычайно важной для спасения человеческой жизни.

## Ключевые слова:

*Staphylococcus aureus*, methicillin устойчивость, посева крови, пункции, PCR