DEVELOPMENT OF A PCR ASSAY FOR IDENTIFICATION OF ANTIBIOTIC RESISTANCE DETERMINANTS AT *Staphylococcus aureus*

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Abstract. The aim of the present study was to develop a PCR assay for identification of antibiotic resistance genes at *Staphylococcus aureus* clinical isolates. Thirty five isolates from the Emergency County Hospital and Clinical Hospital for Infectious Diseases in Cluj-Napoca, Romania, were analyzed. The bacteria were isolated over two years period, in 2007 and 2009 and serotyped at the Clinical Hospital of Infectious Disease, Cluj-Napoca. In order to develop a PCR assay for identification of antibiotic resistance genes at *S. aureus* clinical isolates, the bacterial strains were tested for the presence of the *tst*, *sea*, *mecA*, *femA, ermC* and *ermA* genes using the polymerase chain reaction and gene-specific primers. We succeeded in amplifying *mecA* gene to all isolates that exhibit methicillin resistance. Our result concord 100% with standard culture method but they were obtained in 5 hours instead of four days. In order to increase the efficiency of PCR typing and reduce reagent costs, multiple sets of primers were included in a single reaction tube in a multiplex PCR. We succeeded in elaborating a multiplex PCR for *mecA* and *femA* genes.

Keywords: *Staphylococcus aureus*, antibiotic resistance, PCR

INTRODUCTION

Staphylococcus aureus is a major pathogen that causes a wide spectrum of clinical manifestations, such as wound infections, pneumonia, septicemia, and endocarditis [20]. It is responsible for both nosocomial and community-acquired infections. Methicillin-resistant *S. aureus* (MRSA) is a major cause of nosocomial infections worldwide [1]. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) is an increasing problem [3, 9, 18] predominantly in previously healthy individuals including notable risk groups such as the homeless, those who play close-contact sports, military personnel, men who have sex with men (MSM) and injecting drug users (IDUs). An additional characteristic of CA-MRSA strains potentially explaining their emergence is the carriage of exotoxins [6], in particular Panton-Valentine leukocidin (PVL) [25], which is involved in recurrent skin and soft tissue infections or lethal necrotizing pneumonia [8]. Most of these strains are susceptible to several older but clinically important antibiotics [22].

Methicillin resistance in *S. aureus* is caused by the acquisition of an exogenous gene, *mecA*, that encodes an additional β-lactam-resistant penicillin-binding protein (PBP), termed PBP 2a (or PBP2') [12] The *mecA* gene, which originates from a mobile genetic element (named the staphylococcal cassette chromosome *mec* [SCC*mec*] invariably inserted into the *orfX* gene of methicillin-resistant staphylococci, is the genetic basis of methicillin resistance [14, 20]. Genetic regulatory elements and toxin-expressing genes are virulence factors associated with the pathogenic potential of *S. aureus* [17].

To date, the "gold standard" method for MRSA identification relies on culture [2] and provides results in approximately 4 8 to 72 h. Molecular methods provide alternative approaches have spawned new searches for micro organisms that might play important causal roles in a wide variety of poorly explained acute and chronic diseases. Development of molecular assays for the direct detection of micro organisms has become an actively growing specialty. Advanced laboratory techniques have been sought to rapidly identify staphylococcal isolates and determine antimicrobial susceptibility patterns. In particular, several molecular methods have been described to identify and differentiate staphylococcal isolates [7, 19, 24]. The rapid detection of inpatients carrying methicillin-resistant *S. aureus* (MRSA) has the potential of minimizing MRSA transmission and may even be cost-beneficial [10]. The "same-day detection" of MRSA contributed to the reduction of nosocomial MRSA infections in medical intensive care units when detection was linked with appropriate isolation measures [23].

The aim of the present study was to develop a PCR assay for identification of antibiotic resistance genes at *S. aureus* clinical isolates.

MATERIALS AND METHODS

Clinical isolates. We examined 35 clinical isolates of *S. aureus* from the Clinical Hospital for Infectious Diseases and from the Emergency County Hospital, both from Cluj-Napoca, Romania. The bacterial isolates that originated from different hospital sectors (long-term health care facilities, medical wards and surgical intensive care unit) were collected between November 2007 and October 2009. The *S. aureus* isolates recovered from the broths were screened for methicillin resistance by using oxacillin (6 μg/ml) salt agar screen plates. Confirmation of methicillin resistance was performed by disk diffusion testing with 30μg cefoxitin disks, according to Clinical and Laboratory Standards Institute (CLSI) recommendations [2], and detection of the modified penicillin binding protein (PBP 2') by latex agglutination. MRSA identification was confirmed with the Vitek 2 identification and susceptibility testing cards for gram positives.

S.aureus ATCC 25923., and *Escherichia coli* ATCC 25922 were used as reference strains in susceptibility testing.

Preparation of samples and DNA amplification. We used a direct PCR technique, DNA extraction was skipped and the bacterial cell wall denaturated in the first step of the reaction. One or two bacterial colonies from a plate that was incubated overnight were suspended in 100 ml of sterile water and then diluted to a concentration of approximately 10^6 CFU/ml. 3 µl of this suspension was used as the template for amplification by PCR. Using this technique we can skip the expensive DNA extraction and the self contamination of workers is minimized [13].

PCR protocol

A typical 25-µl PCR mixture contained 2.5 µl 10xPCR reaction buffer, 25 pmol of each primer, 200 µM concentrations of each dNTP, 2 µl $MgCl_2$ 25 mM (2 mM final concentration) 0.75 U of *Taq* polymerase, and 3 µl bacterial suspension. PCR was performed in a Thermocycler, (Gradient Palm-Cycler™ ,Corbett Life Science). The parameters for amplification were as follows: initial denaturation at 94°C for 4 min, 30 cycles of: 1 min each at 94°C, 1 min. at 55-60°C (depending on the primer), 1 min. at 72°C and a final extension step at 72°C for 10 min.

Amplicons have been separated on 1.5 % agarose gel, stained with ethidium bromide. The optimisation of PCR was made after McPherson and Møller, (2001) [16] and Roux, (2003) [21]. The primers (Table 1) were designed with "Pick Primers" programe (http://biotools.umassmed.edu accessed in September 2010) according to the sequences found at NCBI data base.

Table 1. Primers used for PCR amplification.

Target gene	Function	Sequence (5'→3')	Amplicon size (pb)	Accesion no.
mecA	methicillin resistance	GGTAACATTGATCGCAACG/ TTGCCAACCTTTACCATCG	984	NC002951
femA	methicillin resistance	AACAGCTAAAGAGTTTGGTGCC/ CATCACGATCAGCAAAAGCT	647	NC007793
tst	toxic shock toxin	ATGGCAGCATCAGCTTGATA/ TTTCCAATAACCACCCGTTT	350	J02615
sea	enterotoxin	GGATATTGTTGATAAATATAAAGGGAAAAAAG/ GTTAATCGTTTTATTATCTCTATATATTCTTAATAGT	439	DQ6411670
ermA	macrolide-lincosamide-streptogramin B resistance	GAACCAGAAAAACCCTAAAGACAC/ ACAGAGTCTACACTTGGCTTAGGATG	513	NC002952
ermC	macrolide-lincosamide-streptogramin B resistance	ATATCTTTGAAATCGGCTCAGG/ GTGAGCTATTCACTTTAGGTTTAGG	420	NC007792
nucA	termonuclease	GCGATTGATGGTGATACGGTT/ AGCCAAGCCTTGACGAACTAA AGC	267	EF529608
tuf	elongation factor Tu	**1.** GCCAGTTGAGGACGTATTCT/ CCATTTCAGTACCTTCTGGTAA **2.** BAAGAGTTTGATCCTGGCTAG/ TTGACCGTGTCTCAGTTCCA	412 320	DQ414206

RESULTS

We detected *mec*A gene at all MRSA isolates; *femA* gene was also present at MRSA isolates with one exception (Fig. 1, lane 23). We succeeded the co amplification of *mec*A and *femA* genes (multiplex PCR) Amplicons for *sea* gene were obtained at tested isolates (Fig. 2).

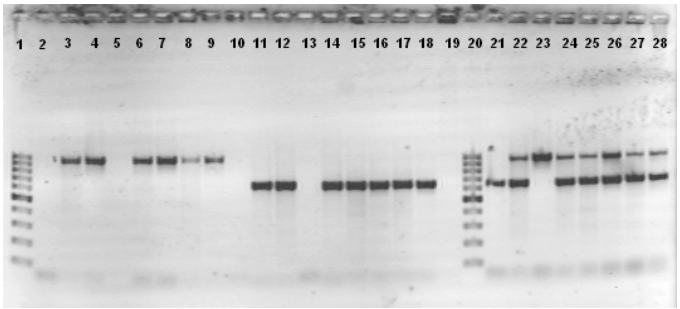

Figure 1. Agarose gel electrophoresis of PCR products after amplification of *mecA* (lanes 2-10) and *femA* (lanes 11-18) genes. Lanes: 1 and 20– molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 21-28 multiplex PCR products for *mecA* and *femA* genes.

The presence of the *tst* gene was not detected at any of the isolates that we tested. In order to be certain that the missing amplicons are due to the missing *tst* gene, we performed the amplification of the *tst* gene and the

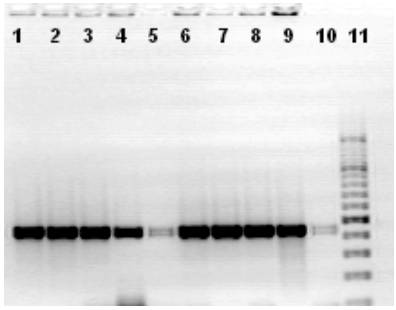

Figure 2. Agarose gel electrophoresis of PCR products after amplification of *sea* gene. Lanes: 11–molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 1-10–different strains of *S. aureus*.

co amplification of the toxic shock toxin gene, with *sea* and *sea* with *nuc* genes, in two multiplex PCR (Fig. 3). This was done in the same conditions and with the same reactivs. The presence of amplicons for the *sea* and *nuc* genes and their absence for the *tst* gene prove the fact that the *tst* gene was missing from the tested isolates.

The results of the *ermA* and *ermC* genes amplification are presented in Fig. 4. We obtained amplicons for one or other of these genes at all the isolates that were resistant to macrolide-lincosamide-streptogramin B. For *tuf* gene we obtained amplicons for both pairs of primers but the second pair of primers gave better results (Fig. 5).

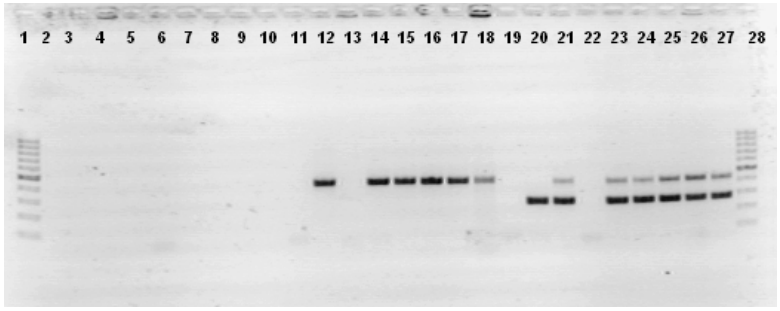

Figure 3. Agarose gel electrophoresis of PCR products after amplification of *tst*, *sea*+*tst* and *nuc*+*sea* genes. Lanes: 1and 28–molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 2-10–*tst*; 11-18-*sea*+*tst*; 20–21 and 23–27-*nuc*+*sea*; 19-negative control (no template).

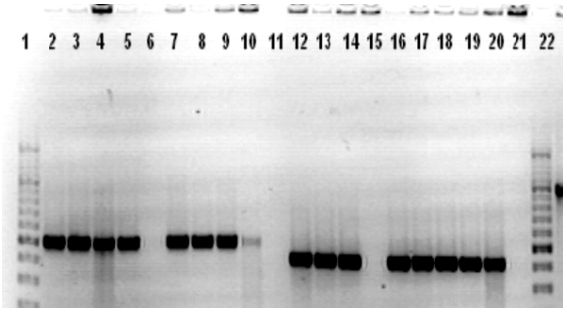

Figure 4. Agarose gel electrophoresis of PCR products after amplification of *ermA* and *ermC* genes. Lanes: 1 and 22-molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 2-11–*ermA*; 12-21-*ermC*.

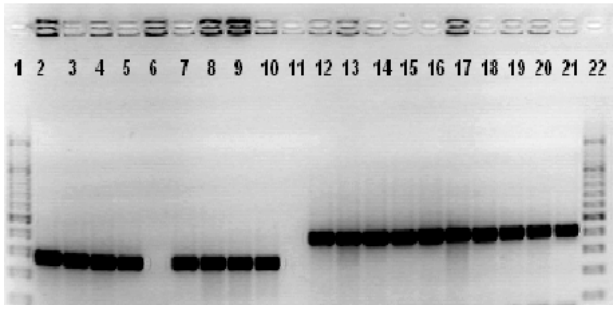

Figure 5. Agarose gel electrophoresis of PCR products after amplification of *tuf* gene. Lanes: 1 and 22- molecular weight marker (O'Range Ruler 100 DNA Ladder, SM1143-Fermentas); 2-11-first pair of primers: 12-21-second pair of primers.

DISCUSSION

In recent years, there has been accumulating evidence that antibiotics, besides their antimicrobial action, potentially have a number of undesired side effects that can, at least in some cases, promote genetic variability of bacteria. In addition to resistant variants, antibiotics have also been shown to select mutator clones, thus stimulating evolution towards further resistance. Furthermore, mutations, recombination and horizontal gene transfer have been reported to be somehow affected when bacteria are exposed to subinhibitory concentrations of certain antibiotics. These findings may have implications for the use of antibiotics, because they may have undesired side effects, such as enhancing antibiotic resistance evolution [5].

The emergence of methicillin resistance in *S. aureus* is of great concern, as MRSA strains are often multidrug resistant. Infections with MRSA are known to be associated with considerable morbidity and mortality [4]. Standard culture methods for the identification of *S. aureus* and the determination of oxacillin susceptibility are time-consuming, usually requiring 2 to 4 days [2]. This delay may lead to the unnecessary use of antimicrobial agents. For these reasons, it has become important to develop rapid diagnostic tests for the detection of MRSA [15].

In order to develop a PCR assay for identification of antibiotic resistance genes at *S. aureus* clinical isolates, thirty five strains were tested for the presence of genes associated with either virulence (*tst*, *sea*,) or antibiotic resistance (*mecA*, *femA, ermC, ermA*) using the polymerase chain reaction and gene-specific primers. This assay is based on the highly conserved *mecA* sequence within all methicillin-resistant strains and species of staphylococci, thus warranting the detection of any organism carrying this resistance determinant [26]. The *femA* gene was selected because encodes a factor which is essential for methicillin resistance and is present in all *S. aureus* isolates. The specificity of MRSA molecular identification is based on the presence of the *mecA* gene and the presence of an *S. aureus*-specific *femA* signal that does not cross-react with other bacterial species, including *S. epidermidis*. We succeeded to amplify *mecA* gene to all isolates that exhibit methicillin resistance. Our result concord 100% with standard culture method but they were obtained in 5 hours instead of four days.

The *tuf* gene, which encodes the elongation factor Tu (EF-Tu), is involved in peptide chain formation and is an essential constituent of the bacterial genome. This fact makes it a target of choice for diagnostic purposes [11]. The *tuf*-based PCR assay developed in this study will be combined, in future experiments in multiplex PCR assays targeting clinically relevant antibiotic resistance genes (e.g., *mecA*).

We also succeeded in elaborating a multiplex PCR for *mecA* and *femA* genes. In order to increase the efficiency of PCR typing and to reduce reagent costs, multiple sets of primers were included in a single reaction tube in a process termed multiplex PCR [15]. The design of the primers was the key strategy in the development of a multiplex PCR assay. Primers must be designed so that they have very close annealing temperature optimums, and the amplification products that they produce need to be of noticeably different sizes to facilitate interpretation. If the amplification products were too close in size, it would be difficult to determine the identity of the amplification product. An additional concern with multiplex PCR was that the mixing of different primers can potentially cause interference in the amplification process, thus making optimization of the reaction difficult, especially as the number of primer pairs in the reaction mixture increases. Other conserved sequences, such as *nuc*, might represent alternate targets in a multiplex PCR. However, multiplex PCR technique was shown to be a fast, practical and economic technique for the detection of methicillin resistant staphylococci.

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