Journal of Medical Bacteriology

Assessment of The Imipenem Antibiotic Effects on The Expression of *Staphylococcus saprophyticus* Serine-Aspartate Repeat Protein-Encoding Genes Isolated from Clinical Cases

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ARTICLE INFO

Article type:
Research Article

Article history:
Received: 02 Oct 2021
Revised: 20 Oct 2021
Accepted: 05 Nov 2021
Published: 24 Nov 2021

Keywords: *Antibiotic Resistance, Imipenem, Staphylococcus saprophyticus, UTI.*

ABSTRACT

Background: *Staphylococcus saprophyticus* is one of the most common causes of urinary tract infections (UTIs). Antibiotic resistance against imipenem is increasing worldwide. Our study aimed to investigate the effect of imipenem on expression of the *S. saprophyticus* serine-aspartate repeat protein-encoding genes by isolating multidrug-resistant strains.

Methods: In this descriptive study, 500 specimens were randomly collected from clinical specimens. Firstly, isolates were identified using standard tests. Then, *S. saprophyticus* species harboring *sdrC, D* and *E* genes were detected using multiplex PCR. Antibiotic susceptibility testing and MIC values of imipenem were performed. the expression of target gene was evaluated by Real time PCR.

Results: Out of 500 samples, 387 *Staphylococcus* species were isolated, among which 155 strains were *S. saprophyticus*. PCR data indicated that 36.77% of *S. saprophyticus* isolates harbored one of *sdr* family genes. The MIC and subMIC values of *S. saprophyticus* species which were treated with imipenem were 125 µg/ml and 225 µg/ml, respectively. Treatment with imipenem induced significant decrease in the expression of *sdrC* and *sdrD* genes as fold changes were -1.241 and -1.322, respectively. There was no statistically significant in *sdrE* gene expression.

Conclusion: The results of our study showed that resistance to imipenem was significantly high in strains harboring *sdrE* gene. Also, treatment with imipenem did not cause any significant change in the expression of *sdrE* gene, which could be a factor in the application of antibiotic resistance by bacteria to this antibiotic. In addition, the present study raises another alarm about the increased risk of antibiotic resistance.

- ***Please cite this paper as:*** Abedin A, Amini K. Assessment of The Imipenem Antibiotic Effects on The Expression of *Staphylococcus saprophyticus* Serine-Aspartate Repeat Protein-Encoding Genes Isolated from Clinical Cases. *J Med Bacteriol.* 2021; **10** (3, 4): **pp**.19-27.

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Introduction

Staphylococcus saprophyticus (*S. saprophyticus*) is a Gram-positive, coagulase negative, cocci in the *Staphylococcus* genus. This microorganism is one of the most common causes of urinary tract infections (UTIs) and one of the important causes of bacteremia in association with vesicular catheters. It is estimated that 10 to 20% of UTI infections are caused by *S. saprophyticus* (1). The emergence of antibiotic resistance is a serious problem for the treatment of patients with staphylococcal infections. According to the studies, 60-85% of staphylococcal strains isolated from clinical specimens are resistant to antibiotics. Coagulase-negative staphylococci (CoNs) are one of the most important pathogens in nosocomial infections. These bacteria have special adhesive properties which cause the organism to tend to attach and to colonize in artificial devices (2).

The most important coagulase-negative staphylococci are *S. saprophyticus* and *S. epidermidis*. These bacteria, which have been considered as the saprophytes for many years, are invasive in recent decades due to the increasing use of medical devices such as catheters and prostheses (3). This has led to the classification of this group of bacteria, especially the two mentioned species, as nosocomial infectious bacteria. Serine-aspartate repeating proteins (Sdr) are members of the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) that contribute to the pathogenesis of *S. saprophyticus*. Sdr proteins interact with host molecules such as β-neurexin, desmoglein 1 (*Dsg 1*) and fibrinogen (4). Overall, these proteins could play manjor roles in *S. aureus* virulence, immune evasion and survival within the host. Beta-lactam antibiotics especially newer generations of cephalosporins are often used to treat most infections caused by coagulase-negative staphylococci (5). However, due to the rapid increase in resistance to this group of medicines, using beta-lactam antibiotics to treat theinfections related to these organisms has been failed. The main mechanism of resistance to beta-lactam antibiotics in staphylococci is divided into two general categories. The first case is the inactivation of penicillin by hydrolysis of the beta-lactam ring (6). The second is the production of beta-lactamase enzymes and Protein Binding Penicillin (PBPs), which reduces the propensity for the drug and leads to greater resistance to semisynthetic penicillin, cephalosporins and carbapenems (7). Beta-lactamases selectively open the beta-lactam loop so that the altered structure of the medicine cannot effectively bind to PBPs, resulting in continued cell wall synthesis. Imipenem is a semi-synthetic carbapenem antibiotic that has a broad-spectrum of antibacterial activity against aerobic or anaerobic gram-positive and gram-negative bacteria, including many resistant species (8). Imipenem is used to treat bacterial infections including endocarditis, pneumonia, UTI, septicemia and skin, bone and joint infections. The antibiotic exerts its antimicrobial action by inhibiting the cell wall synthesis of bacteria by binding to PBPs. Carbapenems, such as imipenem and meropenem, are important medicines with a very broad and stable spectrum of action against beta-lactamases, which are used in the treatment of drug resistance, especially in gram-negative bacteria (9). Recently, many studies show that resistance to imipenem antibiotics is increasing worldwide. Since antibiotic resistance to carbapenems which is known as the last line of treatment for antibiotic resistance is important , this study aimed to investigate the effect of imipenem on expression of the *S. saprophyticus* serine-aspartate repeat

protein-encoding genes by isolating multidrug-resistant strains from clinical cases.

Materials and Methods

Sampling and bacterial isolation

In this descriptive cross-sectional study, 500 specimens were randomly collected from clinical specimens (wound, blood, urine, CSF, catheter, etc.) of patients referring to Shariati hospital, Tehran, Iran. Written consent was obtained from the participants (250 men, 250 women, 17 to 48 years old) in the study in order to use the case information and their samples for research. The names of the people were replaced with codes to remain confidential. All the procedures involving human participants were performed in accordance with the ethical standards of Islamic Azad University, and the 1964 Helsinki declaration and its later amendments.

Isolates were identified using standard microbiological and biochemical methods. At first, samples were cultured on blood agar medium and incubated for 48 hours at 37°C. Isolates underwent biochemical identification using ornithine decarboxylation test, acid production from maltose, trehalose, and sucrose sugars and non-fermentation of glucose under anaerobic conditions, as well as Gram staining, catalase, coagulase, DNase and culture in mannitol salt agar. For short-term storage, isolated strains inoculated to Müller-Hinton agar medium (MHA). Definitive identification of isolates was performed through Gram staining and conventional biochemical tests (ornithine decarboxylation test, acid production from maltose, trehalose, and sucrose sugars and non-fermentation of glucose under anaerobic conditions, catalase, coagulase, DNase and culture in mannitol salt agar) following *16SrRNA* PCR using 27F (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (Reverse primer 5'GGTTACCTTGTTACGA CTT-3') primers.

Multiplex Polymerase chain reaction

Multiplex polymerase chain reaction was used for identification of *S. saprophyticus* isolates harboring *sdr* locus genes. All isolates' genomic DNA was extracted using Iranian Biological Research Center Kit according to manufacturer instructions. the quality and quantity of extracted DNA were checked by the ratio determination of OD 260/280 (NanoDrop 2000c; Thermo Fisher Scientific) and 1.5% agarose gel. Then, the PCR reaction was performed in a final volume of 20 µL containing 4 µL template DNA (200 ng), 10 picomoles of forwarding primer (1 µL), 10 picomoles of reverse primers (1 µL) (Table 1), 4 µL of distilled water and 10 µL of Amplicon 2x mixer. PCR was performed with an initial incubation at 95°C for 10 min followed by 35 cycles of denaturation at 60°C for 60 sec and extension step at 72°C for 1 min. The amplicons were separated by 1.2% agarose gel electrophoresis at 80 V for 2 h. After electrophoresis, fragments were stained by ethidium bromide and visualized by using ultraviolet light.

Antibiotic susceptibility testing

The antibiotic susceptibility pattern was determined by modified Kirby Bauer disc diffusion method against the following antibiotics: cefotaxime (30µg), amikacin (30µg), ceftazidime (30µg), cefepime (30µg), cefoperazone (75µg), sulbactam (10µg), tetracycline (30µg), imipenem (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), levofloxacin (5µg), gentamicin (10µg),

piperacillin-tazobactam (100/10µg) and meropenem (10µg) (MAST Diagnostics, UK). The Mueller–Hinton agar (MHA) plate supplemented with 2% NaCl was swabbed with *S. saprophyticus* culture via a turbidity matching 0.5 McFarland standard and a sterile cotton swab; antibiotics discs were then laid on the surface (CLSI 2018). The MDR (multiple drug resistant), XDR (extensively drug-resistant) and PDR (pan-drug-resistant) were determined based on the guidelines of the Center for Disease Control and Prevention.

Determination of the Imipenem MIC

For estimation of MIC values The micro dilution method was carried out to evaluate the antimicrobial activity. The MIC values were determined on 96-well micro dilution plates according to published protocols (16, 17). To prepare the imipenem stock solution, 10g of imipenem antibiotic (purity>95%, MAST Diagnostics, UK, CAS Number: 64221-86-9) was suspended in one liter of sterile culture medium alsoultrasonic device (Bandelin Sonorex, Germany) was used for proper dispersion for 30 minutes. To prevent errors, suspensions were prepared simultaneously with microbial tests. The minimum inhibitory concentration (MIC) was obtained for each bacteria in contact with the imipenem antibiotic suspension. In this study, the MIC was found to be the lowest concentration of imipenem that inhibits the growth of organisms in culture medium. In this method, a 96-cell microplate with 12 rows, each containing 100 µL of sterile tryptone broth (TSB) medium, was used. Then 100 µL of 2048 to 32 µg/ml concentration of imipenem was added to the rows containing 100 µL of TSB medium. Then 100 µL of 0.5 McFarland *S. saprophyticus* suspension was added to all wells. Also, two rows of wells were used as positive control (TSB and microbial suspension) and negative control of the test (TSB + imipenem). The microplates were then incubated in a shaker incubator (200 rpm, 37 ° C) for a maximum of 24 hours.

Gene expression

The expression levels of *sdr* family and *16SrRNA* (a housekeeping gene to normalize the expression levels of other genes) genes were determined by quantitative real-time PCR (qRT-PCR). The primers used for qRT-PCR are as follow: Forward 5´TACACCGGTCAACATT GAGG3´ and reverse 5´AATTTGATTGCCGCC GTCGG3´ for *sdrC* gene, Forward 5´CCGCGCGCGTGAGATGACCGT3´ and reverse 5´CTCAGTGGCGGTAGAAGAAGG3´ for *sdrD* gene, and Forward 5´AGACAGGAT CGACGTTGCAG3´ and reverse 5´TTGATAAG ATCGAGGGCGTT3´ for *sdrE* gene. Briefly, the total bacterial RNA of the 14 isolates was extracted with RNA extraction Kit (Vivantis, USA) and was quantified by spectrometry (NanoDrop, USA). cDNA was then synthesized using a Prime Script RT Reagent Kit (Cinnagen, Iran) and quantified using SYBR Green (Life Technologies). Ultimately, qRT-PCR was performed with a SYBR Premix Ex Taq II Kit (Cinnagen, Iran) on the thermocycler System (Eppendorf, Hamburg, Germany), with an initial incubation at 94˚C for 120 seconds, followed by 40 cycles of 16 s at 95˚C and 1 min at 60˚C. Relative expression of the target genes was obtained using *16SrRNA* housekeeping gene from *S. saprophyticus*.

Table 1. Primers used to differentiate and isolate Staphylococcus species harboring target genes.

Name	Primer sequence	Bp
sdrC Forward	5'-GAATTACCGATCTATGATGC -3'	355
sdrC Reverse	5'-ACACCTTTGTTAGCACAAAC-3'	355
sdrD Forward	5'-CGTTCACAATCAGTTCTT-3'	211
sdrD Reverse	5'-CCAACTTCTTCTTCCATTTG-3'	211
sdrE Forward	5'-AAC GGG CGT CTC GAT AGA AAA-3' 5'-AAC GGG CGT CCA CAA AATCA-3'	148

The threshold cycle (CT) numbers were confirmed by the detection system softwareand data were analyzed based on ΔΔCt method. The expression levels of target genes were specified and compared with each other. Each reaction was carried out in triplicate, and statistical analysis conducted via SPSS ver.20.

Results

S. saprophyticus isolation

Out of 500 samples, 387 isolates of *Staphylococcus* were detected, among which 155 were identified as *Staphylococcus saprophyticus*. Figure 1 detailed the samples of *S. saprophyticus* species which were isolated. Antimicrobial susceptibility patterns of isolated *S. saprophyticus* species is summarized in figure 2.

PCR results

Extracted DNA quality and quantity were approved using electrophoresis and spectrophotometric method. PCR test was performed on *S. saprophyticus* isolates to identify the existence of *sdr* family genes using specific primers. A standard *S. saprophyticus* (ATCC15305) strain containing the desired genes was considered as positive control. Out of 155 *S. saprophyticus* isolates, 57 isolates (36.77%) harbored one of *sdrC (n=23), E (n=34)* or *sdrD* (n=14) genes (Figure 3).

Antimicrobial activity of imipenem

The MIC and subMIC obtained for *Staphylococcus* species which treated with imipenem were 125 µg/ml and 225 µg/ml, respectively. We examined the effects of imipenem on gene expression, which may reveal the source of antimicrobial activity. *Sdr* family and *16srRNA* genes were explored by using real-time polymerase chain reaction (RT-PCR) following a 24h exposure to varying concentrations of imipenem. According to the findings, imipenem induced suppression in *sdrC* and *sdrD* genes ($p<0.05$). The expression of target genes was suppressed in three samples containing *S. saprophyticus* species treated or not treated with imipenem for *sdrC* and *sdrD* genes ($p=0.002$ and $p=0.0012$, respectively). This indicates the significant decrease in gene expression due to treatment with imipenem. The rate of fold change for *sdrC* and *sdrD* gene in the mentioned groups was -1.241 and -1.322, respectively (Fig 4).

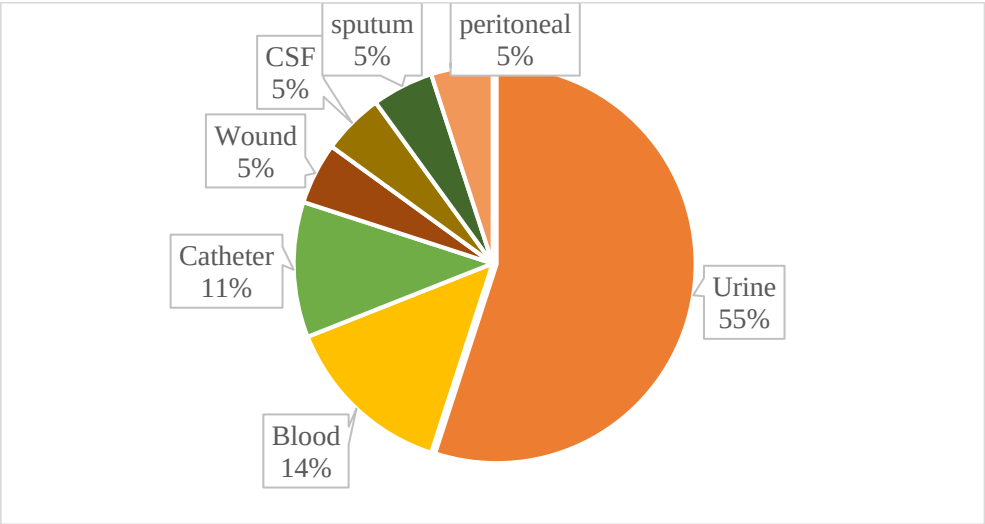

Figure 1. Frequency of samples from which *Staphylococcus saprophyticus* bacteria were isolated in this study (n=155).

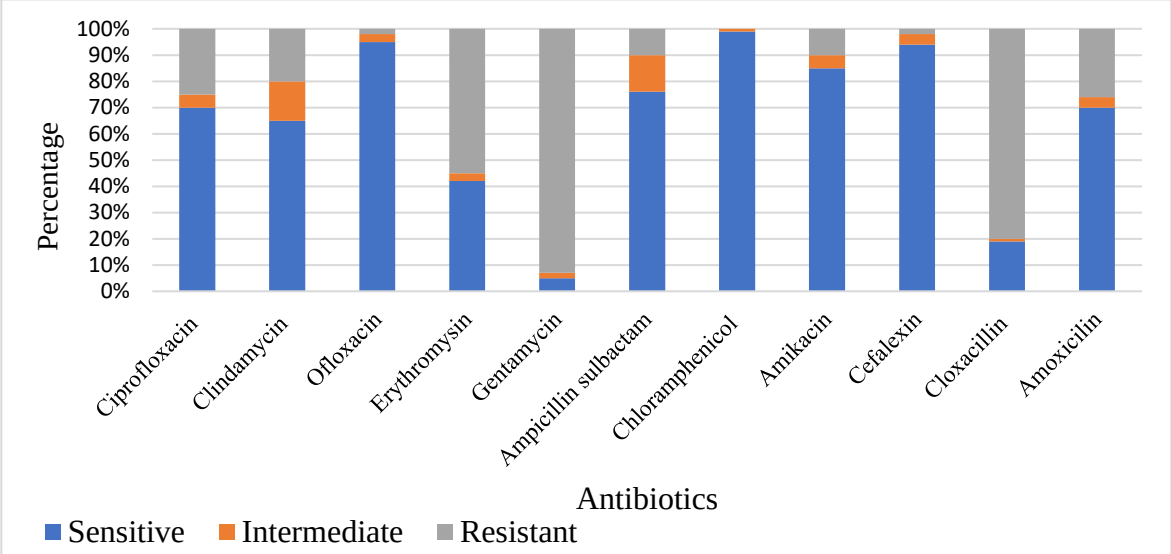

Figure 2. Antimicrobial susceptibility pattern of *Staphylococcus saprophyticus* isolates.

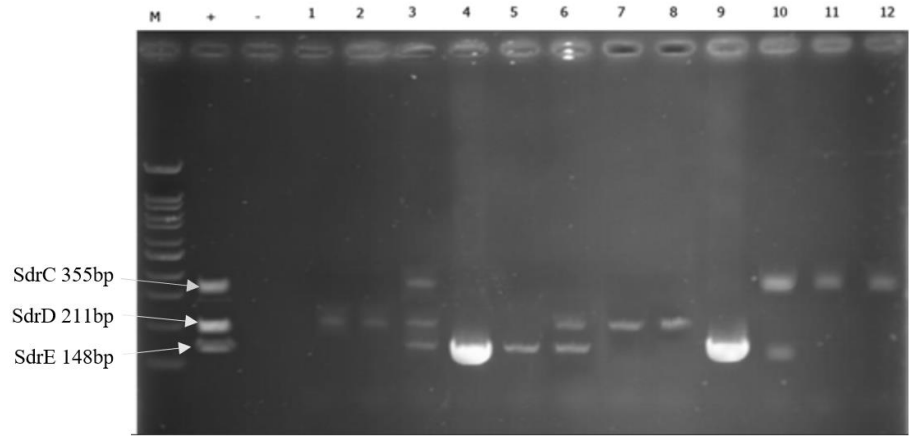

Figure 3. MultiplexPCR products electrophoresis of samples 1 to 12. The M lane:100bp ladder, +: positive control, -: negative control.

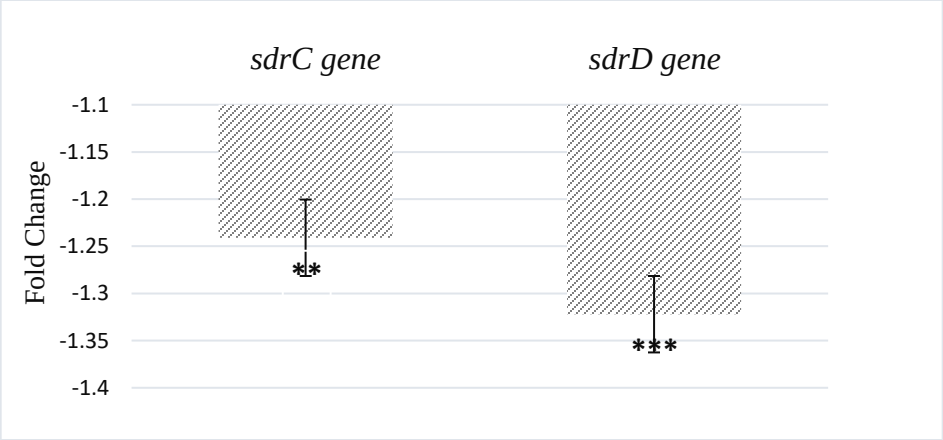

Figure 4. Quantitative real-time PCR showing fold change of *sdrC* and *sdrD* genes in imipenem treated strains that calculated by the $2^{\Delta\Delta CT}$ method. Data represent the averages from three independent experiments. Error bars represent the standard deviations from the means. **: P <0.05; ***: P <0.005 versus control group.

There was no statistically significant in *sdrE* gene expression. The results showed that resistance to imipenem was significantly higher in strains with *sdrE* gene (p<0.001).

Discussion

Staphylococcus saprophyticus is one of the causative agents of urinary tract infections in humans, especially women. In most cases, species isolated from urinary tract infections caused by this microorganism have different resistance genes, some of which play a pivotal role in the pathogenesis of this type of bacterium. Recently, the emergence of new strains with multiple antibiotic resistance genes has brought about problems in the treatment of UTIs caused by these bacteria (10). Urinary tract disease caused by *S. saprophyticus* is a major public health problem

and can pose serious risks. In such cases, antibiotic treatment is life-saving, but with the presence of resistance genes such as *sdr* family and resistance to existing antibiotics, the treatment of these diseases is difficult and it is necessary to evaluate the effectiveness of antibiotics (11). Our data showed that the imipenem significantly reduced the expression level of *sdrC* and *sdrD* genes involved in the pathogenesis of *S. saprophyticus*. The difference among *S. saprophyticus* isolates in terms of existence of *sdr* genes is in accordance with previous studies which reported that the frequency of *sdr* family members could vary remarkably at the isolate level. Our data confirmed that the production of serine-aspartate repeat protein is an isolated specific trait as reported earlier. Many studies have examined the native strains of *Staphylococcus* in Iran. Hashemzadeh et al, studied the biofilm formation, structure and antibiotic resistance in *S. saprophyticus* strains causing UTI in women in Ahvaz, Iran. Their study showed that *S. saprophyticus* isolates were resistant to erythromycin, but all isolates were sensitive to linezolid and vancomycin. In addition, 58 isolates were MDR. Also, their data indicated high rates of antibiotic resistance among *S. saprophyticus* isolates(12). These findings are in line with our data on the emergence of antibiotic resistance in the management of UTIs in Iranian patients. Our sampling was done from Tehran, which has a population context almost equal to that of Iran. The frequency of *S. saprophyticus* in UTIs was 55% which is higher than the reports by Hashemzadeh et al, Nkwocha et al. and Magliano et al (13) (14) (12). The incidence of the *sdr* family genes in *S. saprophyticus* isolates was 36.77%. In previous studies, the prevalence of *sdr* genes among *S. saprophyticus* was 13.4%, 11% and 3.14% for *sdrE*, *sdrC* and *sdrD*, respectively(15). These could be attributed to the source of samples and difference in the population context of the studies. In addition, the data revealed that resistance to imipenem was significantly higher in strains harboring *sdrE* gene.

Conclusion

Our study raises another alarm about the increased risk of antibiotic resistance. It seems that resistance to imipenem in staphylococcal strains is associated with mutations in the *sdrE* gene because a significant decrease in the expression of *sdrC* and *sdrD* genes was observed while there was no change in *sdrE* gene. The data indicated that resistance to imipenem was significantly higher in strains harboring *sdrE* gene. In addition, imipenem was not able to execute notable change in the expression of *sdrE* gene, which may be a sign of antibiotic resistance.

Funding information

This research received no specific grant from any funding agency.

Ethics approval and consent to participate

Not needed.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgment

We are thankful to all members of the Pasargad Laboratory, Tehran, Iran.

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