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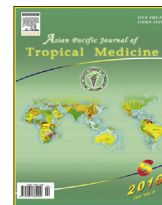

Original research http://dx.doi.org/10.1016/j.apjtm.2016.04.008

The inhibition effect of Chlorpromazine against the β-lactam resistance of MRSA

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ARTICLE INFO

Article history:
Received 15 Feb 2016
Received in revised form 16 Mar 2016
Accepted 8 Apr 2016
Available online 16 Apr 2016

Keywords:
Chlorpromazine
PBP2a
β-Lactamase
mecA
blaZ
MRSA

ABSTRACT

Objective: To investigate the gene related to β-lactam resistance and to confirm the mechanism about a synergy effect between CPZ and β-lactam antibiotics.
Methods: To measure antibacterial activity, we performed a minimum inhibitory concentration (MIC) and synergy test. Transmission electron microscopy (TEM) was used in morphological analysis. To analyze gene expression, we conducted reverse transcriptase polymerase chain reaction (PCR).
Results: We confirmed a synergy effect between CPZ and β-lactam antibiotics. Furthermore, we observed that CPZ affect the cell envelope of MRSA by using TEM. At the gene level, CPZ reduced the expression of resistance genes.
Conclusions: Through this result, we hypothesize that a decrease of resistance factor expressions was caused by CPZ because it disrupts the activity of a sensor protein located in the cell membrane.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) is a significant cause of infections worldwide [1]. This pathogenic organism has been implicated in soft or cutaneous tissue infections, septic arthritis, necrotizing pneumonia, osteomyelitis, and bacteremia [2]. Moreover, MRSA can develop various mutations that confer antibiotic resistance. Penicillin binding proteins (PBPs) are enzymes that catalyze a pentapeptide cross-linking reaction [3,4]. PBPs have a high affinity for β-lactam antibiotics, particularly since these drugs bind the active site of PBPs that are involved in peptidoglycan linking [5]. MRSA produces PBP2a enzymes (encoded by *mecA*) when exposed to β-lactam antibiotics. PBP2a is eventually substituted for PBP in the synthesis of the bacterial cell wall, and it possesses low binding affinities for drugs with β-lactam rings [6]. MRSA also produces a β-lactamase (encoded by *blaZ*) that decreases the activity of β-lactam antibiotics [7].

Several studies have shown that several non-antibiotics exhibit an effect against multidrug resistance [8,9]. Among the non-antibiotics, several phenothiazines were known to be an efficient compound against multidrug resistance. Chlorpromazine (CPZ), which is one of the phenothiazines with an antibacterial effect, was reported to have a synergy effect against MRSA by using it in combination with β-lactam antibiotics [10,11].

The object of this study is to investigate the gene related to β-lactam resistance. Furthermore, we will endeavor to confirm the mechanism about a synergy effect between CPZ and β-lactam antibiotics.

2. Materials and methods

2.1. Bacterial strains and growth condition

Among the four strains used in this research, ATCC 33591 (MRSA) and ATCC 25923 (methicillin-susceptible *S. aureus*, MSSA) were purchased from ATCC (American Type Culture Collection, Manassas, VA). The rest strains are clinical MRSA

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Peer review under responsibility of Hainan Medical College.
Foundation project: This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2012-0004337).

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isolates (DPS-1, DPS-2) obtained from two different patients at Wonkwang University Hospital, Iksan City, Jeollabuk-do, South Korea. The strains were routinely grown at 37 °C with aeration in Mueller–Hinton agar (MHA) and Mueller–Hinton agar (MHB). Methicillin resistance factors (*mecA* and β-lactamase) were confirmed by using the polymerase chain reaction (Table 1).

2.2. Minimum inhibitory concentration (MIC) and synergy effect test

The MIC was determined using the broth microdilution method, in accordance with the Clinical and Laboratory Standard Institute guidelines (CLSI) [12]. Serial two-fold dilutions of CPZ and 10 μL antibiotics in 100 μL MHB were prepared in 96-well microplates. The microplates were injected with MRSA and adjusted to 0.5 McFarland standards (approximately 10 μL of 1.5×10^8 CFU/mL, which was 1.5×10^7 CFU/mL). The MIC was the lowest CPZ concentration in which *S. aureus* growth was suppressed after 24 h incubation at 37 °C. The checkerboard method was used to measure the synergistic effect of CPZ and antibiotics [13]. This assay was executed with CPZ in combination with ampicillin (AM) and oxacillin (OX). The antibiotics were mixed in MHB and serially diluted with CPZ. The MRSA concentration was adjusted to 1.5×10^7 CFU/mL. Growth suppression was checked after 24 h incubation at 37 °C. Each experiment was repeated three times. The interactions between CPZ and antibiotics were determined using the fractional inhibitory concentration index (FICI).

2.3. Transmission electron microscopy (TEM)

The experiments with mechanism were performed by using only MRSA (ATCC 33591) because the strain was a reference strain and was inhibited by CPZ combined with antibiotics. The MRSA of a logarithmic phase were prepared by diluting overnight cultures into MHB and continuing growth at 37 °C until the cultures reached the mid-logarithmic phase of growth. The MRSA of an MHB-grown logarithmic phase were treated with 1/2 MIC and MIC of CPZ for 30 min. Following the treatment, 2 mL of the culture was collected by centrifugation at 13 000 rpm for 10 min. After removing the supernatant, pellets were fixed by immersion in modified Karnovsky fixative solution containing 2% glutaraldehyde and 2% paraformaldehyde in a 0.05 M (pH 7.2) sodium cacodylate buffer [14]. For TEM analysis, the specimens were fixed by a post-fixed solution containing 1% osmium tetroxide in a 0.05 M (pH 7.2) sodium cacodylate buffer for 1.5 h at 4 °C. After post-fixation, the samples were dehydrated in an increasing series of ethanol. And, the mixture of propylene oxide and embed 812 resin were infiltrated into a dehydrated samples, in accordance with Mun *et al.* [15]. Ultrathin sections were obtained in an ultramicrotome with a diamond knife, and collected on 200-mesh grids with Formvar film. Grids were counterstained with uranyl acetate and lead citrate, and examined with an energy-filtering transmission electron microscope at 120 kV (LIBRA 120; Carl Zeiss, Oberkochen, Germany) The electronic signals transmitted were recorded using a 4 k⋯4 k slow-scan charge-coupled device camera (Ultrascan 4000 SP; Gatan, Pleasanton, CA), which was attached to the electron microscope.

2.4. Reverse transcriptase polymerase chain reaction (PCR)

OX was used to confirm the resistance gene expression degree of MRSA because it was used to test the β-lactam resistance profile of MRSA. The MRSA (ATCC 33591) samples were cultured in MHB (OD_{600} 0.35–0.45) and treated with CPZ of various concentrations and OX. The MRSA samples treated with CPZ or OX were incubated for 20 min at 37 °C. The RNA was extracted using easy-RED™ BYF Total RNA Extraction Kit, in conformity with the manufacturer's instructions. Single-strand cDNA synthesis was performed using Quantitact Reverse Transcription Kit, in accordance with the manufacturer's instructions. A PCR was performed using a 20 μL mixture of Lugen™ Sensi 2X PCR Premix (Lugen Sci Co., Ltd., ROK) primer, cDNA sample, and sterilized distilled water. The primer sequences of *mecA*, *mecI*, *mecR1*, *blaZ*, *blaI*, *blaR1*, and *16s rRNA* are represented in Table 2. After the PCR, equal amounts of PCR products (12 μL) were subjected to 1.5% agarose gel electrophoresis. The single bands were visualized by ultraviolet (UV) light.

2.5. Statistical analysis

Data from the experiments are presented as the mean ± SEM. The level of statistical analysis was performed by Student's *t*-test (SPSS. ver. 22) for multiple comparisons. The *P*-values <0.05, <0.005 were considered significant.

Table 1

Determination of the *mecA* gene of the *S. aureus* strains used in the experiment.

S. aureus strain	Class	*MecA* gene	β-Lactamase activity	Antibiotic resistance pattern
ATCC 25923	MSSA	−	−	−
ATCC 33591	MRSA	+	+	AM, OX
DPS-1	MRSA	+	+	AM, OX
DPS-2	MRSA	+	+	AM, OX

+: Positive; −: negative; AM: ampicillin; OX: oxacillin; DPS: staphylococcal strains from the Department of Plastic Surgery, Wonkwang University Hospital.

Table 2

Primer used in this experiment.

Primer	Sequence (5′–3′)
blaZ-F	GATAAGAGATTTGCCTATGC
blaZ-R	GCATATGTTATTGCTTGACC
blaI-F	GCAAGTTGAAATATCTATGG
blaI-R	GAAAGGATCCATTTTCTGTACACTCTCATC
blaR1-F	CATGACAATGAAGTAGAAGC
blaR1-R	CTTATGATTCCATGACATACG
mecA-F	ATGAGATTAGGCATCGTTCC
mecA-R	TGGATGACAGTACCTGAGCC
mecI-F	CTGCAGAATGGGAAGTTATG
mecI-R	ACAAGTGAATTGAAACCGCC
mecR1-F	AAGCACCGTTACTATCTGCACA
mecR1-R	GAGTAAATTTTGGTCGAATGCC
16s rRNA-F	CGTGCCTAATACATGCAAGTC
16s rRNA-R	CCGTCTTTCACTTTTGACCA

Table 3

Synergistic effects of Chlorpromazine (CPZ) combined with β-lactam antibiotics against four *Staphylococcus aureus* strains.

Strains	MIC of CPZ		MIC of AM		FICI	Outcome	MIC of CPZ		MIC of OX		FICI	Outcome
	CPZ	With AM	AM	With CPZ			CPZ	With OX	OX	With CPZ		
ATCC 25923	62.50	62.50	1.95	1.95	2.00	Indifference	62.50	15.60	1.95	0.15	0.3125	Synergy
ATCC 33591	62.50	15.60	1 000.00	250.00	0.50	Synergy	62.50	3.90	250.00	31.25	0.1875	Synergy
DPS-1	62.50	7.80	250.00	31.25	0.25	Synergy	62.50	7.80	125.00	31.25	0.3750	Synergy
DPS-2	62.50	15.60	31.25	7.80	0.50	Synergy	62.50	31.25	15.60	7.80	1.0000	Indifference

The synergistic combinations were investigated using the checkerboard method. The fractional inhibitory concentration indices (FICI) were interpreted as follows: synergy <0.50; partial synergy 0.50–0.75; additive effect 0.76–1.00; indifference >1.00–4.00; and antagonism >4.00.

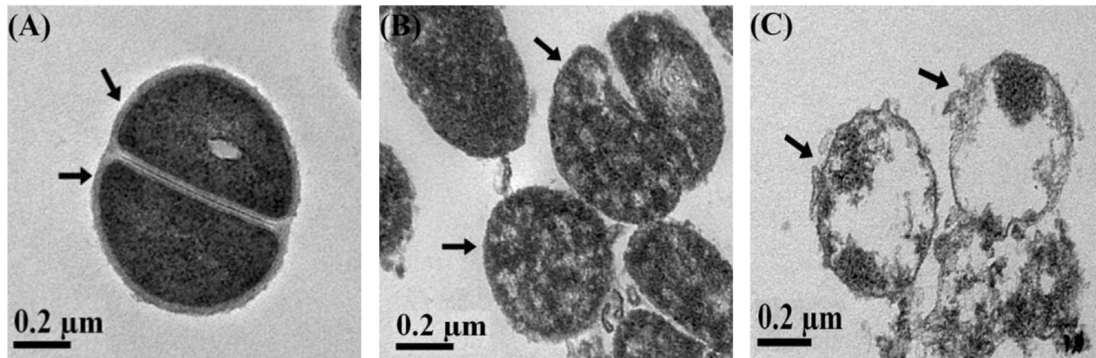

Figure 1. TEM images of MRSA (A, B, C) after 24 h CPZ treatment.
(A) Untreated control MRSA. (B) MRSA treatment with 31.25 μg/mL CPZ. (C) MRSA treatment with 62.5 μg/mL CPZ.

3. Results

3.1. Measurement of MIC and the synergy effect

MICs of CPZ and antibiotics against MRSA and MSSA strains were measured in the broth microdilution method. The MICs of CPZ and antibiotics are represented in Table 3. The growth of the strains was inhibited in concentration of 62.5 μg/mL CPZ. The synergistic effects of CPZ and antibiotics against MRSA and MSSA strains were measured using checkerboard assay. The synergy effects of the CPZ combination with β-lactam antibiotics are represented in Table 3. The results show that CPZ combined with antibiotics inhibited growth of the strains except for one strain, respectively.

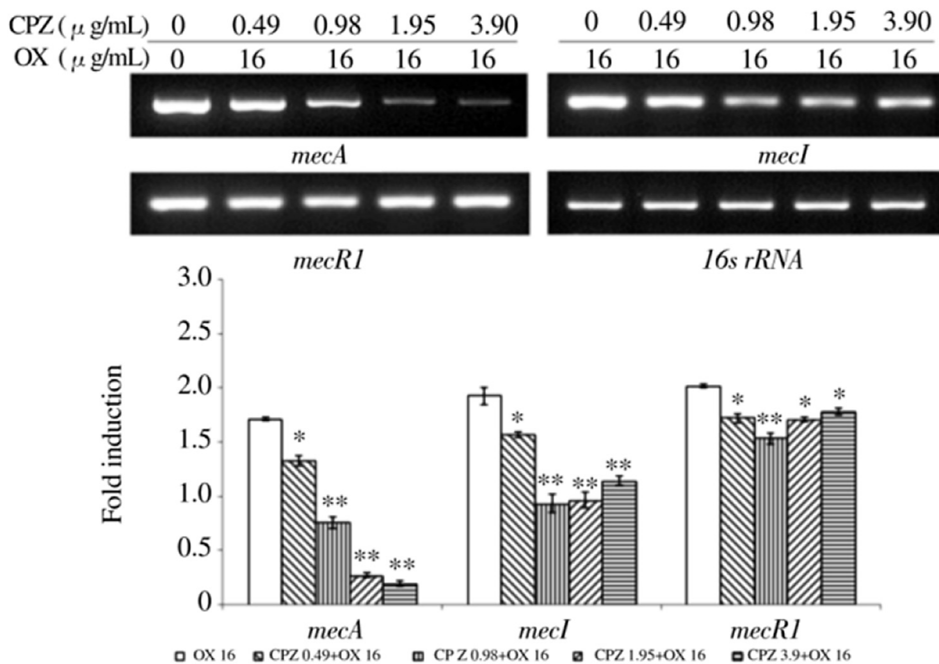

Figure 2. The gene expression related to PBP2a. PCR showing the expression of *mecA*, *mecI* and *mecR1* of MRSA exposed to CPZ of various concentrations in combination with 16 μg/mL oxacillin.
16s rRNA was used as loading control. Expression levels were normalized to *16s rRNA* level. The data are mean ± S.D. for triple-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ as compared to OX alone, were determined.

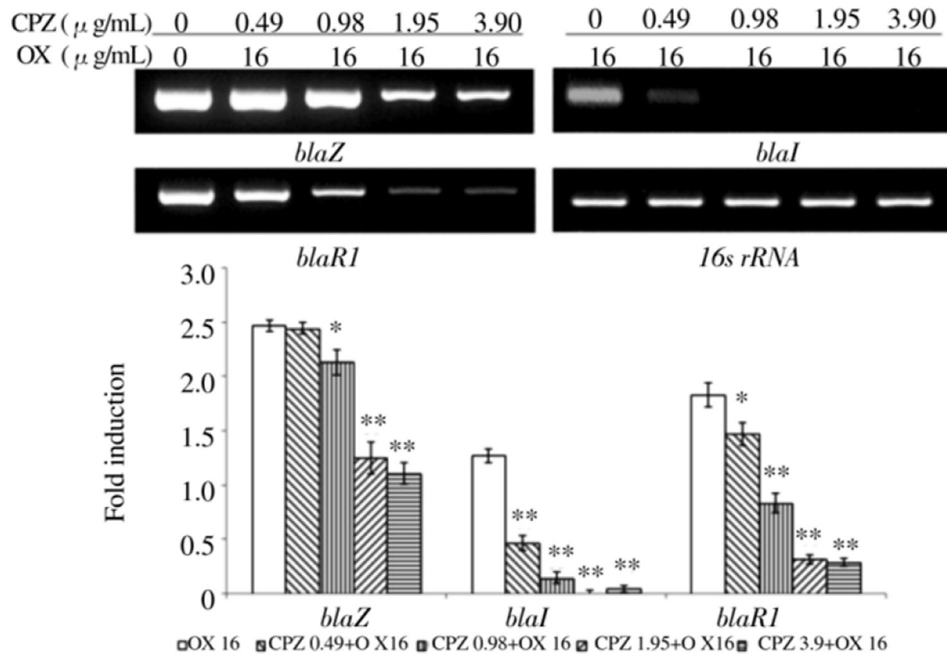

Figure 3. The gene expression related to β-lactamase. PCR showing the expression of *blaZ*, *blaI* and *blaR1* of MRSA exposed to CPZ of various concentrations in combination with 16 μg/mL oxacillin.
16s rRNA was used as loading control. Expression levels were normalized to *16s rRNA* level. The data are mean ± S.D. for triple-independent experiments. $^{*}P < 0.05$, $^{**}P < 0.005$ as compared to OX alone, were determined.

3.2. Observation of cell by TEM

The TEM image of MRSA showed a change of the cytoplasmic membrane and cell wall following exposure to CPZ. The untreated MRSA strains were observed to be intact. Whereas, MRSA strains exposed to 31.25 μg/mL CPZ were observed to change the cell wall. Furthermore, MRSA cells treated with 62.5 μg/mL CPZ were showed to break down the cytoplasmic membrane and cell wall (Figure 1).

3.3. Confirmation of mRNA expression by reverse transcription PCR

At the mRNA level, β-lactam resistance gene expression degree was confirmed by a reverse transcription PCR. In bacteria treated with only oxacillin, resistance gene expression was activated by the antibiotic. But, in bacteria treated with CPZ and oxacillin together, the gene expression mostly displayed a decreased tendency. Especially, when the strain was treated with CPZ, the expression of *mecA* and *blaZ* gene, which were involved in encoding of PBP2a and β-lactamase, showed a concentration dependent reduction. Furthermore, the expression of regulator gene was reduced by CPZ (Figures 2 and 3).

4. Discussion

Infectious diseases by MRSA are a growing problem worldwide. Recently, infections by MRSA pose an important menace to patients [16]. Infections associated with multidrug-resistant bacteria are increasingly difficult to treat because of the decrease of antibiotic efficacy by increased resistance [17]. To solve this problem, the studies on several phenothiazines were conducted by researchers [8,9]. We performed the study on CPZ among phenothiazine that was known to possess antibacterial activity against MRSA [11].

PBP2a and β-lactamase are a major resistance factor against β-lactam antibiotics, such as penicillin and ceftaroline [18,19]. PBP2a and β-lactamase were encoded by *mecA* and *blaZ*, respectively. The transcription of *mecA* and *blaZ* progresses by following a series of processes. When the MRSA was exposed to β-lactam antibiotic, *MecR1* (or *BlaR1*), a sensor-inducer protein located in cell membrane that was encoded by *mecR1* (or *blaR1*), sense the antibiotic and induce cleavage of *MecI* (or *BlaI*), which was a repressor protein. *MecI* (or *BlaI*), encoded by *mecI* (or *blaI*), represses transcription of *mecA* (or *blaA*) because *MecI* (or *BlaI*) binds an operator site upstream of *mecA* (or *blaZ*). Therefore, the transcription of *mecA* (or *blaZ*) was initiated by the cleavage of *MecI* (or *BlaI*). At the same time, the transcription of *mecI* (or *bla I*) and *mecR1* (or *blaR1*) is located in the opposite side to *mecA* (or *blaZ*) start [20–24].

We confirmed that CPZ have an antibacterial activity against MRSA through the measurement of MIC. Furthermore, we verified a synergy effect between CPZ and antibiotics by a checkerboard method. Through this result, we hypothesized that the CPZ affect the susceptibility of β-lactam antibiotics.

To demonstrate a cause about the reduction of β-lactam resistance, we performed morphological analysis, gene analysis, and western blotting analysis. In a TEM image, MRSA, treated with CPZ of 1/2 MIC, was observed to alter a cell envelope and MRSA, treated with MIC, was observed to be undergoing necrosis. This result showed that the CPZ affect a cell envelope of MRSA. We performed gene analysis to confirm how the activity of CPZ on a cell envelope affects the β-lactam resistance factor. In the strains treated with CPZ, the expression of *bla* and *mec* genes showed a decreasing tendency. The *bla* and *mec* genes transcription were important to produce resistance factors such as PBP2a and β-lactamase. Furthermore, this process was initiated by the signal transmission of a sensor-inducer protein located in the cell membrane [20]. The result showed that CPZ affect cell membrane of bacteria. Moreover, we confirmed that gene expression was reduced by CPZ. The data suggests that

CPZ affects a sensor-inducer protein by perturbation of the cell membrane of MRSA. Consequently, this action of CPZ increases the susceptibility of β-lactam antibiotics against MRSA.

Conflict of interest statement

The authors declare there have been no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

Acknowledgment

This study was supported by the Basic Science Research Program through the National Research Foundation (NRF) of Korea funded by the Ministry of Education (NRF-2013R1A1A2064673), 2013060380, the Korea government (MSIP) (2008-0062484), Republic of Korea.

References

[1] Deresinski S. Methicillin-resistant *Staphylococcus aureus*: an evolutionary, epidemiologic, and therapeutic odyssey. *Clin Infect Dis* 2005; **40**: 562-573.

[2] Otto M. Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu Rev Microbiol* 2010; **64**: 143-162.

[3] Fishovitz J, Hermoso JA, Chang M, Mobashery S. Penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *IUBMB Life* 2014; **66**: 572-577.

[4] Scheffers DJ, Pinho MG. Bacterial cell wall synthesis: new insights from localization studies. *Microbiol Mol Biol Rev* 2005; **69**: 585-607.

[5] Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. *Clin Microbiol Rev* 1997; **10**: 781-791.

[6] Berger-Bächi B, Rohrer S. Factors influencing methicillin resistance in staphylococci. *Arch Microbiol* 2002; **178**: 165-171.

[7] Brown DF, Reynolds PE. Intrinsic resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *FEBS Lett* 1980; **122**: 275-278.

[8] Amaral L, Viveiros M, Kristiansen JE. “Non-Antibiotics”: alternative therapy for the management of MDRTB and MRSA in economically disadvantaged countries. *Curr Drug Targets* 2006; **7**(7): 887-891.

[9] Kristiansen JE, Hendricks O, Delvin T, Butterworth TS, Aagaard L, Christensen JB. Reversal of resistance in microorganisms by help of non-antibiotics. *J Antimicrob Chemother* 2007; **59**(6): 1271-1279.

[10] Kaatz GW, Moudgal VV, Seo SM, Kristiansen JE. Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2003; **47**: 719-726.

[11] Kristiansen MM, Leandro C, Ordway D, Martins M, Viveiros M, Pacheco T, et al. Phenothiazines alter resistance of methicillin-resistant strains of *Staphylococcus aureus* (MRSA) to oxacillin *in vitro*. *Int J Antimicrob Agents* 2003; **22**: 250-253.

[12] Clinical and Laboratory Standards Institute. *M11–A8. Methods for antimicrobial susceptibility testing of anaerobic bacteria that grow aerobically*. 8th ed. Wayne, Pa: Clinical and Laboratory Standards Institute; 2009.

[13] Mun SH, Kang OH, Joung DK, Kim SB, Seo YS, Choi JG, et al. Combination therapy of sophoraflavanone B against MRSA: *in vitro* synergy testing. *Evid Based Complement Alternat Med* 2013; **2013**: 823794; http://dx.doi.org/10.1155/2013/823794.

[14] Karnovsky MJ. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J Cell Biol* 1965; **27**: 137A-138A.

[15] Mun SH, Joung DK, Kim SB, Park SJ, Seo YS, Gong R, et al. The mechanism of antimicrobial activity of sophoraflavanone B against methicillin-resistant *Staphylococcus aureus*. *Foodborne Pathog Dis* 2014; **11**(3): 234-239.

[16] Figueroa M, Jarmusch AK, Raja HA, El-Elimat T, Kavanaugh JS, Horswill AR, et al. Polyhydroxyanthraquinones as quorum sensing inhibitors from the guttates of *Penicillium restrictum* and their analysis by desorption electrospray ionization mass spectrometry. *J Nat Prod* 2014; **77**: 1351-1358.

[17] Li M, Du X, Villaruz AE, Diep BA, Wang D, Song Y, et al. MRSA epidemic linked to a quickly spreading colonization and virulence determinant. *Nat Med* 2012; **18**: 816-819.

[18] Otero LH, Rojas-Altuve A, Llarrull L, Carrasco-López C, Kumarasiri M, Lastochkin E, et al. How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. *Proc Natl Acad Sci USA* 2013; **110**: 16808-16813.

[19] Dubée V, Soroka D, Cortes M, Lefebvre AL, Gutmann L, Hugonnet JE, et al. Impact of β-lactamase inhibition on the activity of ceftaroline against *Mycobacterium tuberculosis* and *Mycobacterium abscessus*. *Antimicrob Agents Chemother* 2015; **59**(5): 2938-2941.

[20] Archer GL, Bosilevac JM. Signaling antibiotic resistance in staphylococci. *Science* 2001; **291**: 1915-1916.

[21] Hackbarth CJ, Chambers HF. blaI and blaR1 regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 1993; **37**: 1144-1149.

[22] Zhang HZ, Hackbarth CJ, Chansky KM, Chambers HF. A proteolytic transmembrane signaling pathway and resistance to beta-lactams in staphylococci. *Science* 2001; **291**: 1962-1965.

[23] Sharma VK, Hackbarth CJ, Dickinson TM, Archer GL. Interaction of native and mutant *MecI* repressors with sequences that regulate *mecA*, the gene encoding penicillin binding protein 2a in methicillin-resistant staphylococci. *J Bacteriol* 1998; **180**: 2160-2166.

[24] Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, et al. Genomic basis for methicillin resistance in *Staphylococcus aureus*. *Infect Chemother* 2013; **45**(2): 117-136.