



Effects of Salt Stress on the Antimicrobial Drug Resistance and Protein Profile of *Staphylococcus aureus*

Haleh Ganjian¹, Iraj Nikokar^{2*}, Azita Tieshayar², Ali Mostafaei³, Nour Amirmozafari⁴, Sara Kiani³

¹ Azad University of Lahijan, Guilan, IR Iran

² Laboratory of Microbiology and Immunology of Infectious Diseases, Paramedicine Faculty, Guilan University of Medical Sciences, Guilan, IR Iran

³ Kermanshah University of Medical Sciences, Kermanshah, IR Iran

⁴ Tehran University of Medical Sciences, Tehran, IR Iran

ARTICLE INFO

Article type:
Original Article

Article history:
Received: 01 Mar 2011
Revised: 20 Apr 2011
Accepted: 01 May 2011

Keywords:
Staphylococcus aureus
Salt Stress
Antibiotic Resistance
Electrophoresis
Polyacrylamide Gel

ABSTRACT

Background: *Staphylococcus aureus* is the causative agent of a high percentage of nosocomially acquired infections and food-borne illnesses. Antimicrobial resistance of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), continues to be a concern for clinicians worldwide.

Objectives: The aim of this study was to investigate the effects of salt stress on the antimicrobial drug resistance and protein profile of *S. aureus*.

Materials and Methods: *Staphylococcus aureus* (ATCC 25823) was grown in trypticase soy broth at 37°C. Cells in the exponential growth phase were gradually exposed to sub-lethal salt stress with concentrations ranging from 5% to 35% (wt/vol). Thereafter, these cells were harvested and re-suspended in a tube containing 0.5 mL of saline. To standardize the number of bacteria, the bacterial suspension was compared to the 0.5 McFarland standard suspension. Antibiotic susceptibility was determined using the disk diffusion method, and the method involved plating of cell suspensions with stressed cells and unstressed cells on Mueller-Hinton agar plates. The pooled proteins from each condition were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Results: Compared to the unstressed cells, the cells exposed to salt showed significant changes in resistance to rifampicin ($P=0.032$), penicillin ($P=0.02$) and methicillin ($P=0.001$). Furthermore, SDS-PAGE analysis of pooled proteins from cells exposed to salt showed changes in the protein profile.

Conclusions: We conclude that salt stress is responsible for the changes in protein profile and antimicrobial resistance of *S. aureus*, especially to methicillin.

©2012, AJUMS. Published by Kowsar M.P.Co. All rights reserved.

► Implication for health policy/practice/research/medical education:

The results of this study indicate that the use of high amount of salt in food preservation can lead to the development of antibiotic-resistant population of *S. aureus* as a food-borne pathogen.

► Please cite this paper as:

Ganjian H, Nikokar I, Tieshayar A, Mostafaei A, Amirmozafari N, Kiani S. Effects of Salt Stress on the Antimicrobial Drug Resistance and Protein Profile of *Staphylococcus aureus*. *Jundishapur J Microbiol.* 2012; 5(1):328-31.

1. Background

Staphylococcus aureus is one of the important human pathogens involved in food-related diseases and a common cause of community-associated infection (1, 2). This organism proliferates in food and releases one or more heat-stable enterotoxins, causing food-borne illnesses (3). *S.*

* Corresponding author: Iraj Nikokar, Laboratory of Microbiology and Immunology of Infectious Diseases, Paramedicine Faculty, Guilan University of Medical Sciences, P.O. Box: 44715-1361, Langeroud, IR Iran. Tel: +98-1425237070, Fax: +98-1425237171, Email: Nikokariraj@yahoo.com, Nikokariraj@gums.ac.ir

aureus is the most common cause of infections in hospitalized patients and has been a major concern for well over a century (4). The spectrum of diseases caused by this organism is extremely wide, ranging from superficial infections to deep-seated and systemic infections such as pneumonia, endocarditis, osteomyelitis, and sepsis (5). The treatment of staphylococcal infections has become extremely challenging due to the propensity of the organism to rapidly evolve into antibiotic-resistant strains. Antibiotic resistance is an emerging problem worldwide, and widespread use of antibiotics is likely to be the main reason for the increase in antibiotic resistance (6).

Prevalence of resistant *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), is increasing in clinical settings. MRSA is one of the most common causes of nosocomial infections. Methicillin, a semi-synthetic penicillinase-resistant penicillin, was introduced in 1960 for the treatment of penicillinase-producing strains of *S. aureus*; methicillin-resistant strains of *S. aureus* were identified in 1961 (7). Environmental stresses, including temperature, pH, salts, osmotic pressure, and alkaline and acidic conditions can affect the growth rate and population of bacteria (8). To our knowledge, stress and antimicrobial drug resistance has been studied more extensively in Gram-negative bacteria such as *Escherichia coli*; however, very little is known about these factors with regard to *S. aureus*.

2. Objectives

The aim of this study was to investigate the effect of environmental stress on the antibiotic susceptibility and protein profile of *S. aureus* as a prototypical Gram-positive bacterium.

3. Materials and Methods

S. aureus (ATCC 25823) was obtained from the Iranian Research Organization for Science and Technology and grown in trypticase soy broth (TSB; Merck) at 37°C. *S. aureus* cells in the exponential growth phase were exposed to sub-lethal salt stress by using concentrations ranging from 5% to 35% (wt/vol). The stress-treated cells were then harvested by centrifugation (3,000 ×g for 15 min) and were re-cultured on mannitol salt agar (MSA, Merck). Bacteria were re-suspended in a tube containing 0.5ml of saline. To standardize the number of bacteria, 0.5 McFarland standard was prepared by adding 99.5ml of 1% sulfuric acid to 0.5ml of 1.175% BaCl₂ solution. Spectrophotometric analysis of the bacterial suspension by using 0.5 McFarland standard revealed that the suspension contained 1.5×10⁸ bacteria per milliliter (CFU/mL). Cell suspensions containing stressed and normal bacterial cells were plated on Mueller-Hinton agar (MHA; Merck Company) plates using a sterile swab. Susceptibility to 11 antibiotics was tested by performing the disk diffusion method by using commercial disks (MAST Diagnostics, Merseyside, UK) according to the Clinical Laboratory Standards Institute guidelines (9).

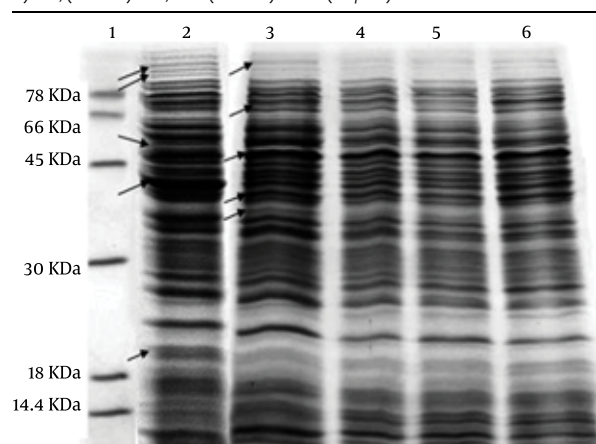
The antibiotics used and their disk potencies were as follows: erythromycin, 15µg; penicillin G, 10U; gentamicin,

10µg; ciprofloxacin, 5µg; cefalexin, 30µg; chloramphenicol, 30µg; co-trimoxazole, 25µg; rifampicin, 5µg; clindamycin, 2µg; cephalothin, 30µg; and methicillin (25µg). The plates were incubated at 37°C for 17 to 24 h and were then examined 4 times for the development of zones of inhibition in each lawn growth around the disc. The pooled protein from stressed and non-stressed (control) bacterial cells were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (10). Statistical analyses were performed by ANOVA by using Statistical Package for Social Sciences (SPSS, version 11.5). A *P* value of ≤0.05 was considered statistically significant.

4. Results

S. aureus ATCC 25823 cultures were gradually adapted to the 4 salt concentrations (5.0%, 15%, 25%, and 35.0% [wt/vol]), in various periods ranging from 5 to 20 min. Evaluation of antimicrobial drug resistance pattern revealed significant differences in zone sizes between the test and control suspensions under salt stress. Salt-stressed *S. aureus* suspensions had significantly smaller inhibition zones in the presence of rifampicin, penicillin, and methicillin. However, the zone of gentamicin was bigger to those observed for un-stressed control suspensions (Table). The tested bacterial strain had the highest resistance to methicillin (*P*<0.001), significantly higher than that shown to any other antibiotics tested, especially in 35% concentration of salt. The SDS-PAGE analysis of pooled proteins at 4 conditions, (i) 5.0%, (ii) 15%, (iii) 25%, and (iv) 35.0% (wt/vol) of salt, in comparison with the control, is shown in Figure 1. Comparison of the protein profiles between control and those treated with different salt concentrations showed that salt treatment decreased the intensity of some of the bands (20 kDa, 40 kDa, and 60 kDa) at salt concentrations of 5% to 35%. In contrast, some protein bands (47 kDa, 66 kDa, and >78 kDa) showed increased intensity in the same range of salt concentrations.

Figure 1. SDS-PAGE Analysis of Pooled Proteins From (Lane 2) Un-Stressed (Control) *S. aureus* and Stressed under 4 Conditions, (Lane 3) 5.0%, (Lane 4) 15%, (Lane 5) 25%, and (Lane 6) 35.0% (wt/vol) of Salt.



The marker is in Lane 1. The intensity of the bands (20 kDa, 40 kDa, and 60 kDa) decreased from salt concentrations varying from 5% to 35%, and compared to the control, the intensity of other protein bands (47 kDa, 66 kDa, and >78 kDa) increased under similar conditions.

Table. Anti-Microbial Drug Resistance Pattern Observed in Stressed and Un-stressed Varieties of *S. aureus* by Using the Disk Diffusion Method

	Mean Zone Size, mm				Non-Stressed	P value
	Stressed With Salt					
	5%	15%	25%	35%		
Rifampicin	1.77	1.70	1.95	2.30	2.50	0.032
Penicillin G	1.32	2.10	2.00	2.4	3.20	0.002
Clindamycin	2.30	2.27	2.25	2.25	2.10	0.298
Gentamicin	2.20	2.17	2.20	2.20	1.90	0.049
Erythromycin	2.40	2.32	2.22	2.00	2.20	0.502
Co-trimoxazole	2.40	2.20	2.20	2.15	2.10	0.818
Ciprofloxacin	2.40	2.37	2.37	2.35	2.40	0.430
Cefalexin	2.17	2.35	2.52	2.70	2.20	0.219
Chloramphenicol	1.70	1.90	2.02	2.30	1.90	0.601
Methicillin	2.22	2.20	2.00	1.72	3.20	0.001

5. Discussion

Historically, salt has been used both as an additive and preservative in foods, and abundant information on it can be found in the literature. salt has often been incorporated as an antimicrobial agent in meat, meat products, or brine solutions (11). In the present study, we showed that *S. aureus* could grow gradually at 4 salt conditions, (i) 5. 0%, (ii) 15%, (iii) 25%, and (iv) 35. 0% (wt/vol). This finding is consistent those of some previous reports (12, 13). Osmotolerance in this organism is therefore an interesting topic for several reasons. The intracellular concentration of potassium (K) is high and does not change much on salt stress since osmoprotectants can enter *S. aureus* cells through various transport mechanisms that are activated or induced by salt stress. *S. aureus* can also activate some genes and express proteins in response to salt stress (14-16). In the present study, antibiotic susceptibility in *S. aureus* ATCC 25823 decreased in the presence of sub-lethal concentrations of salt. A similar response has been reported in wild-type strains of *S. aureus* isolated from commercial food kitchens (8).

Genotypic changes may be responsible for the development of hyper-resistant varieties of *Staphylococcus*. By point mutations, target sites for antibiotic binding can be inactivated and heterogeneous population can be generated with increased spontaneous mutation rates (hypermutable strains) (8, 17, 18). Our results show that salt stress causes significantly greater changes in methicillin resistance than in any other antibiotics tested. The mechanism behind the staphylococcal resistance to methicillin is attributable to the expression of a unique penicillin-binding protein, PBP2a, which has a much lower affinity for beta-lactam antibiotics (19). Analysis of protein profile in this study shows changes in the protein, which was attributable to the expression and inhibition of some important proteins.

Exposure of microorganisms to sub-lethal concentrations of salt can induce the expression of stress proteins with a profile similar to that of stress protein expression induced by heat shock. Many environmental stresses can induce the Mar (multiple antibiotic resistances) operon that is known

to regulate the expression of a large number of genes, including the efflux pump (the arcAB efflux pump) (8, 20). This suggests that using high concentrations of salt in food preservation can lead to the development of population or subpopulation of *S. aureus* with decreased susceptibility to antibiotics. Results of this study demonstrated that sub-lethal salt stress could significantly alter the antibiotic resistance and protein profile of *S. aureus*. We conclude that the increased use of salt in food processing may contribute to the development and dissemination of antibiotic resistance *S. aureus* as food-borne pathogens.

Acknowledgements

None declared.

Financial disclosure

None declared.

Funding/Support

This article is a compilation of results of MS thesis (Code, 20230507882015) from Azad University of Lahijan, Guilan, Iran. The tests were conducted at the Laboratory of Microbiology and Immunology of Infectious Diseases, Paramedicine Faculty, Gilan University of Medical Sciences, Langeroud.

References:

1. Fridkin SK, Hageman JC, Morrison M, Sanza LT, Como-Sabetti K, Jernigan JA, et al. Methicillin-resistant *Staphylococcus aureus* disease in three communities. *N Engl J Med*. 2005;**352**(14):1436-44.
2. Normanno G, La Salandra G, Dambrosio A, Quaglia NC, Corrente M, Parisi A, et al. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *Int J Food Microbiol*. 2007;**115**(3):290-6.
3. Balaban N, Rasooly A. Staphylococcal enterotoxins. *Int J Food Microbiol*. 2000;**61**(1):1-10.
4. Ekrami A, Samarbafzadeh A, Alavi M, Kalantar E, Hamzeloi F. Prevalence of methicillin resistant *Staphylococcus* species isolated from burn patients in a burn center, Ahvaz, Iran. *Jundishapur J Microbiol*. 2011;**3**(2):84-91.
5. Bertini G, Nicoletti P, Scopetti F, Manoocher P, Dani C, Orefici G.

- Staphylococcus aureus* epidemic in a neonatal nursery: a strategy of infection control. *Eur J Pediatr*. 2006;**165**(8):530-5.
6. Lietzau S, Sturmer T, Erb A, Von Baum H, Marre R, Brenner H. Prevalence and determinants of nasal colonization with antibiotic-resistant *Staphylococcus aureus* among unselected patients attending general practitioners in Germany. *Epidemiol Infect*. 2004;**132**(4):655-62.
 7. Rahbar M, Safadel N. Evaluation of cefoxitin disk diffusion test for routine detection of methicillin-resistant *staphylococcus aureus*. *Iran J Pathol*. 2006;**1**(4):145-8.
 8. McMahon MA, Xu J, Moore JE, Blair IS, McDowell DA. Environmental stress and antibiotic resistance in food-related pathogens. *Appl Environ Microbiol*. 2007;**73**(1):211-7.
 9. Sader HS, Ferraro MJ, Reller LB, Schreckenberger PC, Swenson JM, Jones RN. Reevaluation of Clinical and Laboratory Standards Institute disk diffusion breakpoints for tetracyclines for testing Enterobacteriaceae. *J Clin Microbiol*. 2007;**45**(5):1640.
 10. Nikokar I, Kajbaf M, Mak VM, Farajzadeh A, Kamali E, Mostafaei A, et al. Isolation and purification of Ag 85 complex from Mycobacterium Bovis (Bcg) and assessment of its cell proliferation response in whole blood. *Yakhteh (The Cell)*. 2004;**6**:144-150.
 11. Hajmeer M, Ceylan E, Marsden JL, Fung DY. Impact of sodium chloride on *Escherichia coli* O157:H7 and *Staphylococcus aureus* analysed using transmission electron microscopy. *Food Microbiol*. 2006;**23**(5):446-52.
 12. Tsai M, Ohniwa RL, Kato Y, Takeshita SL, Ohta T, Saito S, et al. *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiol*. 2011;**11**:13.
 13. Vilhelmsson O, Miller KJ. Synthesis of pyruvate dehydrogenase in *Staphylococcus aureus* is stimulated by osmotic stress. *Appl Environ Microbiol*. 2002;**68**(5):2353-8.
 14. Arney DR, Phillips CJ. The effects of changes in sodium and potassium concentration on growth of mastigenic bacteria in vitro. *Intern J Appl Res Vet Med*. 2005;**3**:242-8.
 15. Scybert S, Pechous R, Sitthisak S, Nadakavukaren MJ, Wilkinson BJ, Jayaswal RK. NaCl-sensitive mutant of *Staphylococcus aureus* has a Tn917-lacZ insertion in its ars operon. *FEMS Microbiol Lett*. 2003;**222**(2):171-6.
 16. Vijaranakul U, Nadakavukaren MJ, Bayles DO, Wilkinson BJ, Jayaswal RK. Characterization of an NaCl-sensitive *Staphylococcus aureus* mutant and rescue of the NaCl-sensitive phenotype by glycine betaine but not by other compatible solutes. *Appl Environ Microbiol*. 1997;**63**(5):1889-97.
 17. Livermore DM. Bacterial resistance: origins, epidemiology, and impact. *Clin Infect Dis*. 2003;**36**(Suppl 1):S11-23.
 18. Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. *Antimicrob Agents Chemother*. 2000;**44**(7):1771-7.
 19. Japoni A, Alborzi A, Orafa F, Rasouli M, Farshad S. distribution patterns of methicillin resistance genes(mecA) in *staphylococcus aureus* isolated from clinical specimens. *Iran Biomed J*. 2004;**8**(4):173-8.
 20. Alekshun MN, Levy SB. Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli* marRAB locus, by multiple chemicals in vitro. *J Bacteriol*. 1999;**181**(15):4669-72.