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

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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 concernfor 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% to35% (wt/vol). There after, these cells were harvested and re-suspended in a tube containing 0.5mL 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 profileand antimicrobial resistance of *S. aureus*, especially to methicillin.

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.

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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.*

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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

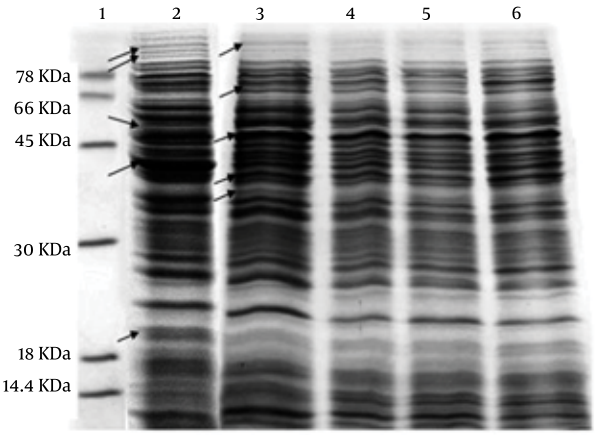
S. aureus (ATCC 25823) was obtained from the Iranian Research Organization for Science and Technology and grown in trypticase soy broth (TSB; Merck) at 37°C. *S. aureus* cells in the exponential growth phase were exposed to sub-lethal salt stress by using concentrations ranging from 5% to 35% (wt/vol). 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% $BaCl_2$ solution. Spectrophotometric analysis of the bacterial suspension by using 0.5 McFarland standard revealed that the suspension contained 1.5×10^8 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 to11 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 4times 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 wereperformed by ANOVA by using Statistical Package for Social Sciences (SPSS, version11.5). 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 zonesin 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 (47kDa, 66 kDa, and >78kDa) 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					
	Stressed With Salt				Non-Stressed	*P* value
	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 populationcan 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.

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