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Relationship Between Antibiotic Resistance with *Spa* Gene Polymorphism Coding Protein A and its Typing with PCR-RFLP Technique in *S. aureus* Isolated from Foodstuffs

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Abstract

Background: *Staphylococcus aureus* is an important cause of hospital and community acquired infections. Food borne diseases are an important problem in public health. Protein A is a protein 42 KDa, which is expressed in all strains of this bacterium.
Objectives: This study aimed to evaluate the relationship between antibiotic resistances with *spa* gene polymorphism.
Methods: A total of 1,050 food samples were collected during 8 months in Hamedan, Iran. Food samples were evaluated for the presence of *spa* genes of *S. aureus*. The antibiotic susceptibility testing was performed using disk diffusion agar. After extraction of genomic DNA, *nuc* and *spa* genes were detected. Finally, with the PCR-RFLP method, *spa* typing was performed. The relationship between the antibiotic resistance rate and *Spa* types were analyzed by the SPSS software.
Results: Results showed that the 98 cases (9.33%) of *S. aureus* were isolated. The most frequent resistance was observed against tetracycline (8.41%). *Spa* gene was reported in all isolates and 4 different patterns of *spa* gene was seen. Furthermore, a significant correlation between different strains isolated from diverse foodstuffs and different patterns of *spa* ($P < 0.05$) was also found. In addition, the relationship between resistance to different antibiotics with obtained types showed that there is a significant correlation between resistance to erythromycin ($P = 0.014$) and clindamycin ($P = 0.016$) with different *spa* types.
Conclusions: In regards to the increased resistance to antibiotics in strains isolated from foodstuffs, rapid and accurate typing of *S. aureus* to identify transmission of the infectious organisms is very important. Molecular typing of *Spa* protein can prevent epidemics and reduce the infections and costs of nosocomial infections.

Keywords: Antibiotic Resistance, *spa* Gene

1. Background

Food-borne diseases are considered as a major public health problem, and annually by spending billions of dollars, millions of people in the world infected and a part of them may be hospitalized or expired (1, 2). *S. aureus* have more than 20 different species, which are scattered in different habitats. Some of them there are in the skin, glands, mucous membranes of animals and humans, as well as transport to the animal products such as; milk, meats, and environmental resources such as; soil, sand, dust, air, and natural waters (3). Pathogenicity in *S. aureus* depends on the expression of a wide variety of secreted molecules associated with the cell wall of bacteria and escape from the host immune system and responses of host tissue (4, 5).

Protein A 42-kDa of *S. aureus* is a protein associated with the cell wall, which is expressed in all strains of this bacterium (5). Protein A is a major component in the cell wall of *S. aureus*. The sequence analysis of x region of *spa* gene coding protein A of *S. aureus* has shown that this region is composed of repeats with 24 base pair. The binding region to FC of immunoglobulins is called x region, which with study of this region, determined the difference between epidemic and non-epidemic isolates (6-8). Polymorphism in this gene, due to the different length of this gene in different strains, is about 1,150 to 1,500 bp. Another factor for polymorphism of *spa* gene is due to repetitive sequences in X area. So, PCR-RLFP *spa* gene is used for typing of *S. aureus*.

The *spa* typing method for *S. aureus* has a high accuracy and discrimination, of course, it has some problems such as; need to sequencing, which is costly as well as changes in the x region of *spa* gene be considered while other changes will be ignored (9). Fast and accurate typing of *S. aureus* to identify transmission of the infectious organism is very important. With molecular typing of this protein can to short or prevent of epidemics and reduce the number of infections and costs resulted from nosocomial infections (10).

2. Objectives

The present study aimed to evaluate the relationship between antibiotic resistance with *spa* gene polymorphism coding protein A and its typing with PCR-RFLP technique in *S. aureus* isolated from foodstuffs.

3. Methods

3.1. Sample Collection

In this cross-sectional study, during a period of 8 months, 1,050 food samples (creamy, meat, milk, yogurt, butter, and cheese) were randomly collected from different parts (Hamedan, Malayer and Asadabad) of the Hamedan province. Each of the samples in sterile containers with special bolts were dumped in minimum time while maintaining cold conditions was transferred to the microbiology laboratory of Hamedan University of Medical Sciences.

3.2. Isolation of S. aureus Isolates

After, homogenization of samples in sterile conditions, with the help of saline at room temperature, were entered into the enrichment process and with help of enrichment broth (Quelab) containing 3.5% Potassium Tellurite) for *S. aureus* incubated for 24 hours at 37°C. Then, samples were cultured in Bird Parker agar medium (Merck, US) containing 5% egg yolk and tellurite and incubated at 37°C for 48 hours. Colonies with a black appearance and with a bright halo were considered as positive as well as cultured on the blood agar medium again, and for all of isolates in addition to the gram staining, catalase, coagulase, DNase tests, mannitol fermentation test using mannitol salt agar medium (QUELAB), and VP test were performed (11). *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 25923™) in all steps was used as a positive control.

3.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed for all isolates using the Kirby Bauer disk diffusion method. Antimicrobial susceptibility of *S. aureus* isolates was determined using gentamicin (10 μg), tetracycline (30 μg), trimethoprim/sulfamethoxazole (25 μg), ciprofloxacin (5 μg), erythromycin (15 μg), clindamycin (2 μg), rifampin (5 μg), and cefoxitin (15 μg) antibiotics (MAST Laboratories Ltd., Bootle, Merseyside, UK) according to the guidelines of the clinical and laboratory standards institute (CLSI), (12).

3.4. DNA Extraction and PCR Performance

DNA of *S. aureus* isolates were extracted based on the kit direction (Bioflux, Japan). Then, the extracted DNA was stored in the freezer at -20°C. To identify *nuc* and *spa* genes, 2 μL of extracted DNA was added to 18 μL of PCR reaction mixture (final volume 20 μL). The primers sequences for genes were as follows; F- *nuc*: 5-GCGATTGATGGTGATACGGTT-3; R- *nuc*: 5 AGCCAAGCCTTGACGAACTAAAGC-3 (13), F-*spa*:5-ATCTGGTGGCGTAACACCTG-3; R- *spa*:5-CGCTGCACCTAACGCTAATG-3 (14).

Fast, Simultaneous, and Sensitive Detection of Staphylococci

The PCR cycle program for *nuc* gene was as follows: 1 cycle for initial denaturation at 94°C for 5 minutes, 35 cycles with denaturation at 94°C for 30 seconds, annealing stage in 55°C at 55 seconds, elongation step at 72°C for 1 minute and final elongation stage in 72°C for 10 minutes; also for *spa* gene, the program was as follows: Initial denaturation: 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing temperature; 55°C for 1 minute, extension step; 72°C for 1.5 minutes, 35 cycles and final extension; 72°C in 5 minutes (2, 14).

3.5. Performing of RFLP Technique

PCR products were digested in one sterile micro-tube and 1 μL of Rsa I restriction enzyme was added to 1 μL of PCR products. Then, 2 μL of enzyme buffer 10x was added, and finally, 16 μL of distilled water was added to the final volume (20 μL), and then micro-tubes containing the mixture were incubated in 37°C overnight. After this time, microtubes were incubated for 20 minutes at 50°C for deactivation of the enzyme. In the next step, enzymatic digestion products were electrophoresed for 2 hours on agar gel 2% in a voltage of 90 volts (15).

4. Results

4.1. Frequency of S. aureus Isolates in Different Foodstuffs

Overall, in this study, 1050 samples of foodstuffs were considered for contamination with *S. aureus* isolates. A to-

tal of 98 strains (9.33%) were isolated. Isolates were confirmed by PCR using *nuc* gene as an indicator to detection of *S. aureus*.

4.2. Results of Antimicrobial Susceptibility Testing

Among the 98 isolates of *S. aureus* that was detected in this study, the most antibiotic resistance was observed to tetracycline with 41 cases (41.8%), erythromycin with 38 cases (38.8%), and gentamicin with 36 cases (36.7%), respectively. Our results also showed that *S. aureus* isolates from foodstuffs had very little resistance to cefoxitin 6 cases (6.1%), which representing the resistance to methicillin antibiotic. The resistance to other antibiotics was as follows; clindamycin with 26 cases (26.5%), ciprofloxacin with 32 cases (32.7%), rifampin with 25 numbers (25.5%), and finally, trimethoprim-sulfamethoxazole with 13 cases (13.2%).

4.3. The Results of PCR for spa Gene Encoding the Protein A

The sequence analysis in the X region of *spa* gene of *S. aureus* showed that this region is composed of repeats with a 24 base pair. In this study, several different gene amplicons in the different samples were detected, which is shown in Table 1. Also, the results of electrophoresis of PCR products are presented in Figure 1.

4.3.1. The Results of RFLP PCR for spa Gene Encoding the Protein A

After PCR, products were cut using restriction enzyme RsaI. The PCR- RFLP patterns of *spa* gene products were different. The frequencies are shown in Table 2.

4.4. The Relationship Between Various spa types with Different Patterns of Antibiotic Resistance

After the end of RFLP and determination of types, the relationship between them and the resistance to different antibiotics was determined by statistical analysis (Table 3).

5. Discussion

S. aureus is one of the most important gram-positive bacteria, which have numerous roles in production of infections and is one of the outstanding indicators of nosocomial infection. In addition, this bacterium is one of the 4 most common and important causes of food poisoning (11). Prevalence of *S. aureus* in this study was 9.3%, which was similar to the study conducted by Soltan Dallal et al. from Tehran, which the prevalence of 9.5% has been reported (16). A study carried out by Ali aydin et al. from Turkey (2011), stated that the prevalence of *S. aureus* in foodstuffs was13.8% (17). As well as, in a similar study conducted by Crago et al. from Canada, during 2007 to 2010, the prevalence rate of 10.53% was reported (18).

In the current study, contamination between dairy products and meat with a prevalence rate of 62 (9.24%) and 36 (9.49%), respectively, had no significant difference in bacterial abundance ($P > 0.05$). However, in the study carried out by Soltan Dallal et al. there was a significant difference between the frequency of bacteria isolated from dairy products (17%) and meat products (5/3%), ($P < 0.05$), (16). In contrast with the present study, a research from Italy (2014), the prevalence of *S. aureus* in milk and dairy products was reported 39% (19), which is higher than our obtained results. The other research study from Turkey by Nurhan Ertas et al. (2010), have reported the prevalence of 57% (20). The reason for this high rate is likely due to receiving the raw milk from cows or sheep with mastitis disease.

Other pollutants factors are contaminated equipment, as well as personnel. In addition, production of raw milk and unpasteurized cheese (cottage cheese) are important concerning factors for development of food poisoning. In this study, the high resistance was observed to tetracycline, erythromycin, and gentamicin antibiotics with frequency of 41 (41.8%), 38 (38.8%), and 36 (36.7%), respectively. These findings were similar to the results obtained of other studies in Iran, which were conducted on strains isolated from foodstuffs (3). Statistical analysis showed that there was a significant difference between different antibiotic resistances in strains isolated from diverse foodstuffs sources ($P < 0.05$). In total, in comparison of antibiotic resistance in strains that have been isolated from foodstuffs with strains isolated from hospitals, it determined that nosocomial isolates have a much higher proportion of resistance than obtained isolates from the community (21). Therefore, it can be concluded that the isolates exist in the community if entered in the hospital; they can acquire resistance to different antibiotics through genetic elements.

As already mentioned, after the PCR, 6 patterns were observed, which among those, pattern (1300 bp) was the most prevalent. RFLP-PCR results showed that 4 different types of *spa* there were in isolates, named as A to D. Most of strains isolated from different foodstuffs types have pattern B (1200 - 1300 bp), respectively. By use of statistical analysis, a significant relationship between different strains isolated from diverse foodstuffs and diverse patterns of *spa* ($P < 0.05$) was found.

In a study carried out by Mahmudi et al. in Hamdan, on 200 *S. aureus* isolated from clinical samples and carriers, after cutting enzymes, have identified 4 different *spa* types (21), which were in agreement with obtained results and types of foodstuffs in present study. However, this could indicate that the strains isolated from one geographic region have almost identical patterns. Afrough et al. showed

Table 1. The Results of PCR for *spa* Gene in Samples[a]

PCR Product	Cream	Yogurt	Milk	Red Meat	Chicken Meat	Cream Milk	Cheese	Bucher	Total
Bp1000	-	-	1 (1.02)	-	-	-	1 (1.02)	-	2 (2.04)
Bp1100	1 (1.02)	-	-	1 (1.02)	1 (1.02)	-	1 (1.02)	3 (3.06)	7 (7.14)
Bp1200	1 (1.02)	1 (1.02)	7 (7.14)	7 (7.14)	3 (3.06)	-	6 (6.12)	1 (1.02)	26 (26.53)
Bp1300	2 (1.96)	1 (1.02)	12 (12.24)	9 (9.18)	5 (5.10)	-	5 (5.10)	-	34 (34.71)
Bp1400	-	-	7 (7.14)	6 (6.12)	2 (2.04)	2 (2.04)	6 (6.12)	2 (2.04)	25 (25.52)
Bp1500	-	-	2 (2.04)	1 (1.02)	-	-	-	-	3 (3.06)
Total	4 (4.08)	2 (2.04)	29 (29.06)	25 (25.52)	2 (11.22)	2 (2.04)	19 (19.38)	6 (6.12)	89 (100)

[a] Values are expressed as No. (%).

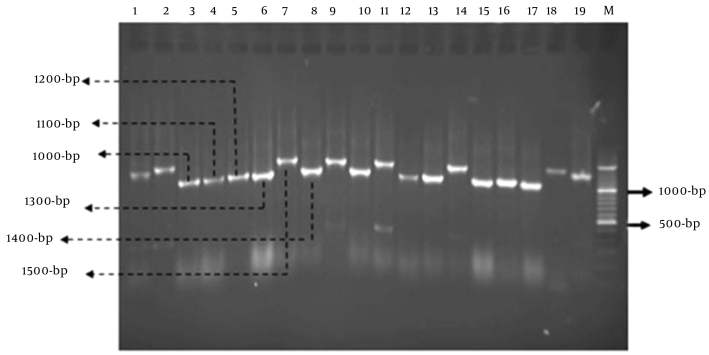

Figure 1. Electrophoresis of *spa* Gene Products

Table 2. The Results of PCR-RFLP for *spa* Gene Products in *S. aureus* Isolates

PCR Products	PCR-RFLP Pattern	Genotype	(%) No.
Bp1000-1100	Bp240-850	A	(9.2) 9
Bp1200-1300	Bp600-700	B	(57.1) 56
Bp1400-1500	Bp600-240-850	C	(29.6) 29
bp	Uncutted	D	(4.08) 4

Table 3. Relationship Between Antibiotic Resistances with Different *spa* Types

Antibiotics	A	B	C	D	Total Resistance, %	P Value
Cefoxitin	1	3	2	-	6 (6.1)	0.061
Erythromycin	2	16	16	4	38 (38.8)	0.014
Tetracycline	4	17	16	4	41 (41.8)	0.124
Gentamicin	2	22	12	0	36 (36.7)	0.134
Clindamycin	2	11	13	0	26 (26.5)	0.016
Ciprofloxacin	4	14	12	2	32 (32.7)	0.443
Rifampin	3	12	10	0	25 (25.5)	0.270
Trimethoprim/sulfamethoxazole	2	4	6	2	13 (13.26)	0.151

9 different patterns of *spa* gene including: 650 bp, 800 bp, 900 bp, 1200 bp, and 1400 bp, which were similar with PCR products obtained from this study (10). In addition, this indicated, the existence of genetically similar strains in this area or even in the countries. The frequency of different *Spa* types of *S. aureus* isolated from different foodstuffs in this study is statistically significant (P = 0.009). In addition, the relationship between resistance to different antibiotics with obtained types showed that there was a significant correlation between resistance to erythromycin (P = 0.014) and clindamycin (P = 0.016) with diverse *spa* types.

6. Conclusions

In regards to the increased resistance to antibiotics in strains isolated from foodstuffs, rapid and accurate typing of *S. aureus* to identify transmission of the infectious organisms is very important. Molecular typing of *Spa* protein can prevent epidemics and reduce the infections and costs of nosocomial infections.

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Footnote

Conflict of Interest: None declared.

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