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Prevalence of virulence genes, *agr* and antimicrobial resistance of *Staphylococcus aureus* isolated from food and dairy products in Hamadan, Iran

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ABSTRACT

Staphylococcus aureus is one of the most important foodborne pathogen in food products. Exfoliative toxins (ETs) and toxic shock syndrome toxin-1(TSST-1) are important virulence factors that facilitate bacterial attack and reproduction in the body of host. Expression of exfoliative toxins controlled by accessory gene regulator (age) locus. Therefore, the identification of these genes in Staphylococcus aureus in food samples of very necessary and important. The aim of this study was prevalence of virulence genes (tsst1 and ETs) and agr of Staphylococcus aureus isolated from food products and association of these genes with antibiotic resistance. Between 2013 and 2014, 1050 food samples of various origin purchased and collected in Hamadan province in Iran. The S. aureus strains were isolated from raw milk and dairy products (n= 671) and raw meats (n=379). Samples were examined for the presence of S. aureus. Strains were characterized using standard microbiological procedures. Molecular identification of S. aureus strains confirmed by PCR. All isolates were screened for tsst1, agr, eta genes by PCR amplification. Out of 1050 samples, 98 (9.33%) samples were positive for S. aureus, including 36 (9.49%) of 379 raw meats and 62 (7.2%) of 671 raw milk and dairy products. The most frequent resistance was observed to erythromycin (30.6%), followed by tetracycline (29.6%), Gentamicin (27.6%), Clindamycin (26.5%), Ciprofloxacin(24.5%), Rifampin (24.5%), Sulfamethoxazole/ Trimethoprim (14.3%), and Cefoxitin (6.1%). The TSST-1 was identified in 30.61 percent of isolates, while the eta and etd were found in 63.26and 75.51 percent of isolates, respectively. The distribution of agrA and agrC genes among the 98 food isolates were 63.26 and 14.28 percent, respectively. The detection of the high prevalence rate of virulence genes in this study indicates a potential risk for causing animal originated food poisoning that is a serious problem for public health. Infected animals and acquisition of infection during the processing stage are the main causes of contamination with S. aureus. Therefore, continuous surveillance is essential for monitoring of pathogens that are capable of causing food poisoning.

Keywords: Staphylococcus aureus, TSST-1, ETs, age, Antibiotic resistance

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INTRODUCTION

The percent of the diseases due to foodborne pathogens remains largely unknown and approximately two thirds foodborne illness were caused by unknown agents**[1, 2]**. *Staphylococcus aureus* is one of the most important foodborne pathogen in food products**[3]**. This pathogen produce various infections from relatively mild to more severe diseases**[4]**. These infections very difficult to treat due to the development of drug resistance, especially methicillin-resistant *S. aureus* (MRSA)**[5]**. In addition, this microorganism has enough potential to contaminate animal products such as meats, milk and dairy products**[6, 7]**.

S. aureus produces many toxins such as toxic shock syndrome toxin 1 (TSST-1) and exfoliative toxins (ETs) that facilitate bacterial attack and reproduction in the body of host**[8]**. TSST-1 is considered to be a superantigen that role in the Staphylococcal Toxic Shock Syndrome (TSS)**[9]**. TSS is characterized by high fever, headache, confusion, subcutaneous oedema, rash, desquamation, diarrhea, vomiting and hypotension, that resulting in multiple organ failure**[10]**. Secreted superantigens at mucosal sites such as vagina and nasopharynx lead to a massive release of cytokines including tumor necrosis factor (TNF), interleukin-2 (IL-2) and IFN-γ which are responsible for development of the typical clinical signs**[11]**. Exfoliative toxins(ETs), responsible for staphylococcal scaldedskin syndrome(SSSS)**[12]**. The genes encoding ETs (*eta*, *etb*, and *etd*) are located on mobile genetic elements (MGEs) such as prophases, plasmids or transposons**[13, 14]**. Transfer of these genes through MGEs involve in distribution of pathogenic strain of *Staphylococcus aureus* and emergence of super bugs**[15]**. ETs have serine protease activity and cleave a peptide bond in the extracellular region of desmoglein 1 (Dsg1) and facilitating bacterial skin invasion**[16]**. In *S. aureus* the expression of most virulence factors and surface proteins controlled by accessory gene regulator (*agr*) locus which encodes a two-component system that down-regulate surface proteins and up-regulate secreted proteins**[17]**. Based on study of Foster in 1987, expression of gene encoding ETA(*eta*) in S. aureus is dependent on *agr* locus**[18]**.It seems that *agr* locus have a major role in human infection. It has been suggested that changes in the secretion of virulence factor expression can lead to acquisition of antibiotic *resistance in S. aureus***[19-21]**.

The aim of this study was prevalence of virulence genes (*tsst1*, *agr* and *eta*) of *Staphylococcus aureus* isolated from food products and association of these genes with antibiotic resistance.

MATERIALS AND METHODS

2.1. Food samples and bacterial strains

Between 2013 and 2014, 1050 food samples of various origin purchased and collected in Hamadan province in Iran. The *S. aureus* strains were isolated from raw milk and dairy products (n= 671) and raw meats (n=379). Samples were examined for the presence of *S. aureus*. Strains were characterized using standard microbiological procedures (Gram-staining, hemolytic activity on sheep blood agar, catalase production, oxidase test, growth in Baird–Parker agar and coagulase tube test). In addition, molecular identification of *S. aureus* strains confirmed by PCR.

2.2. Antimicrobial susceptibility testing

The antibiotic susceptibility pattern of all isolates was determined by disk agar diffusion method with the zone diameters measured at 24 h according to the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2013). Mueller Hinton agar was used for antimicrobial susceptibility test. The 8 antibiotic discs (Mast, UK) were Cefoxitin (30µg), Ciprofloxacin (5µg), Clindamycin (2µg), Erythromycin(15µg), Gentamicin(30µg), Rifampicin(5µg), Trimethoprim/ Sulphamethoxazole(1.25/23.75µg) and Tetracycline (30µg). Strain ATCC 25423 was used as positive control.

Interpretation of results were applied according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

2.3. DNA extraction and PCR for tsst1, agr and eta genes

Total DNA was extracted from overnight-grown pure isolates using the DNA extraction Kit (BioFlux Co., Tokyo, Japan), according to the manufacturer's instructions. Extracted DNA stored at 40˚C for use in PCR amplification. All isolates were screened *tsst1*, *age*, *eta* genes by PCR amplification. All primer sequences have been listed in Table 1. The genes encoding *agr* (*agrA*, *agrB*, *agrC* and *agrD*), toxic-shock syndrome toxin-1 (*tsst1*) and exfoliative toxin genes (*eta* and *etd*) were detected by PCR. PCR products were visualized by UV transillumination after electrophoresis on 1.0% agarose gels.

Table 1: Oligonucleotide primers used in PCRs

Gene	Sequences	Product (bp)	References
Nuc	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	279	**[22]**
TSST-1	F: TTCACTATTTGTAAAAGTGTCAGACCCACT R: TACTAATGAATTTTTTTATCGTAAGCCCTT	180	**[23]**
eta	F: ACTGTAGGAGCTAGTGCATTTGT R: TGGATACTTTTGTCTATCTTTTTCATCAAC	190	**[23]**
etd	F: GAATTAAGTAGTACCGCGCTAAATAATATG R: GCTGTATTTTTCCTCCGAGAGT	492	**[23]**
agrA	F: GTCACAAGTACTATAAGCTGCGAT R: GTATTACTAATTGAAAAGTGCCATAGC	440	**[24]**
agrB	F: GTCACAAGTACTATAAGCTGCGAT R: GTATTACTAATTGAAAAGTGCCATAGC	572	**[24]**
agrC	F: GTCACAAGTACTATAAGCTGCGAT R: CTGTTGAAAAAGTCAACTAAAAGCTC	406	**[24]**
agrD	F: GTCACAAGTACTATAAGCTGCGAT R: CGATAATGCCGTAATACCCG	588	**[24]**

2.4. Statistical analysis

All data analyses were performed using the statistical software SPSS16. Statistical analysis was performed using the Pearson Chi-squared test to calculate the strength of association between the virulence genes and origin of the isolates, as well as between virulence genes and antibiotic susceptibility patterns. A value of $P<0.05$ was considered significant.

RESULTS

3.1. Isolation and identification of S. aureus

Out of 1050 samples, 98 (9.33%) samples were positive for *S. aureus*, including 36 (9.49%) of 379 raw meats and 62 (7.2%) of 671 raw milk and dairy products. Among food products, traditional cheese with 19(11.17%) isolates from 170 samples and cream with 2(4.25%) isolates from 47 samples had the highest (19) and the lowest *S. aureus* infection, respectively. For molecular confirmation of *S. aureus* strains, PCR amplification for *nuc* gene was performed. All 98 strain carried *nuc* gene.

3.2. Identification of TSST-1, eta, agr genes

DNA of isolates was examined for the presence of *TSST-1*, *eta*, *agr* genes. The *TSST-1* was identified in 30.61 percent of isolates, while the *eta* and *etd* were found in 63.26 and 75.51 percent of isolates, respectively. The distribution of *agrA* and *agrC* genes among the 98 food isolates were 63.26 and 14.28 percent, respectively. Results had shown in Table2.

Table 2: Prevalence of *TSST-1*, *eta* and *agr* genes in *S. aureus* isolated from food products

Types of samples	Sample size No.(%)	No of *S. aureus* isolates (%)	Type of genes				
			TSST-1	***eta***	***etd***	***agrA***	***agrC***
Milk	271(25.8)	29(10.7)	10(10.2)	16(16.32)	24(24.48)	16(16.32)	6(6.12)
Cheese	170(16.19)	19(11.17)	6(6.12)	14(14.28)	14(14.28)	14(14.28)	4(4.08)
Yogurt	45(4.28)	2(4.44)	0(0)	2(2.04)	0(0)	2(2.04)	0(0)
Cream	66(6.28)	4(6.06)	2(2.04)	2(2.04)	2(2.04)	0(0)	2(2.04)
Skim	47(4.47)	6(12.76)	0(0)	2(2.04)	2(2.04)	2(2.04)	0(0)
Butter	72(6.85)	2(2.77)	2(2.04)	4(4.08)	4(4.08)	2(2.04)	0(0)
Meat	243(23.14)	25(10.28)	8(8.16)	14(14.28)	20(20.4)	16(16.32)	2(2.04)
Chicken	136(12.95)	11(8.08)	2(2.04)	8(8.16)	8(8.16)	10(10.2)	0(0)
Total	1050(100)	98(9.33)	30(30.61)	62(63.26)	74(75.51)	62(63.26)	14(14.28)

3.3. Antimicrobial susceptibility testing

A total 98 (9.33%) *S. aureus* strains from 1050 food samples were isolated. Among the 98 isolates, resistance to Erythromycin (30.6%) was the most frequently observed, Tetracycline (29.6%), Gentamicin (27.6%), Clindamycin (26.5%), Ciprofloxacin and Rifampin (24.5%), Trimethoprim-Sulfamethoxazole (14.3%), and Cefoxitin (5.1%). Results had shown in Table3.

Table 3: Results of Antimicrobial susceptibility testing

Antibiotics	Sensitive No.(%)	Intermediate No.(%)	Resistant No.(%)
Cefoxitin	93 (94.9)	nd	5 (5.1)
Erythromycin	65 (66.3)	3(3.1)	30 (30.6)
Tetracycline	68 (69.4)	1(1.0)	29 (29.6)
Gentamicin	71 (72.4)	nd	27 (27.6)
Clindamycin	71 (72.4)	1(1.0)	26 (26.5)
Ciprofloxacin	74 (75.5)	nd	24(24.5)
Rifampin	70 (71.4)	4(4.1)	24(24.5)
SXT	82 (83.7)	2(1.0)	14 (14.3)

nd: not detected

3.4. Statistical analysis

The statistical analysis showed that there was a significant relationship between origin of the isolates and *agrA* gene as well as between origin of the isolates and resistance to Erythromycin, Tetracycline, Clindamycin, Gentamicin, Ciprofloxacin, Rifampin and SXT($p<0.05$). A statistically significant correlation was found between some antibiotic susceptibility patterns and carrying virulance genes in examined isolates. This included between Erythromycin and carrying *eta* genes, between Clindamycin and carrying *agrA* genes, and between SXT and carrying *eta* genes($p<0.05$).

DISCUSSION

Exfoliative toxins(ETs), toxic shock syndrome toxin-1(TSST-1) can horizontally transfer between the strains and accessory gene regulator (agr) have a significant role in expression of gene encoding ETA(*eta*) in *S. aureus***[25]**. Food products have an important role in transfer and dissemination of these genes. The aim of this study was to determine the prevalence of *TSST-1*, *eta* and *agr* genes and antibacterial susceptibility pattern of *S. aureus* isolated from raw meats, raw milk and dairy products in Hamadan, Iran.

In our study, 9.33% of samples were contaminated by *S. aureus*; similar to a study performed in Tehran and Turkey, which reported contamination rates of 9.5% and 13.8%, respectively**[26, 27]**. In the other study, higher contamination rates of *S. aureus* have reported**[28]**. These discrepancies may be due to differences in ecological origin of the strains, cheese production process, number of colonized food handlers, level of hygiene and transport systems.

In this study the TSST1 was found in 30.61% of the *S. aureus* isolates. However, there are some studies with lower frequency (11%)**[29]**. In another study, the *tsst1* gene not found in any of the strains of the isolates**[12]**.

ETA, ETB, and ETD are three main human active exfoliative toxins that responsible for staphylococcal scalded skin syndrome. In our study, prevalence of *eta* and *etd* genes in the isolates were 63.26 and 75.51, respectively. None of the *S. aureus* isolates contained *etb* genes. These results were inconsistent with previous studies. In study of Minghui et al. *etb* and *etd* were not found in any isolate and *eta* was only detected in one isolate of 117 *S. aureus* food isolates**[12]**. In study of Aydin et al. also none of the *S. aureus* isolates contained *eta* and *etb* genes**[27]**.

Karsten et al investigated 429 isolates of *staphylococcus aureus* and *eta* and *etb* gene were identified in 1.2 and 0.5 percent of them, respectively**[30]**.

In the study carried out in Japan, it was demonstrated that ETA was present in 40 percent of strains, while 25 percent of cases contained ETB, and in five percent of them both genes were observed**[31]**.

Moreover, in the study carried out by Osamu et al eighty-eight isolates were positive for *eta* alone, 25 were positive for *etb* alone, and 85 were positive for both genes**[32]**.

These finding obtained by various studies conducted in this area stresses that the prevalence of these genes are different in different geographical regions.

For detection of *agr* types, we used four pair's oligonucleotide primers. Among the *agr* genes, the *agrA* (75.51%) was the most prevalent followed by *agrC*. All isolates were negative for *agrB* and *agrD* genes. These results were similar to previous studies that *agrA* was prevalent in the meat and milk samples**[33, 34]**. In another study, all strains were non-typeable for *agr* locus**[35]**. This discrepancy may be due to geographic distribution and origin of the samples.

CONCLUSION

S. aureus is the most frequently isolated bacterium that causes many cases of food poisoning in the world. The detection of the high prevalence rate of virulence genes in this study indicates a potential risk for causing animal originated food poisoning. Increase of these genes in food products is a serious problem for public health. Infected animals and acquisition of infection during the processing stage are the main causes of contamination with *S. aureus*. Therefore, continuous surveillance is essential for monitoring of pathogens that are capable of causing food poisoning.

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