

Antibiotic Resistance and Detection of *femA* Gene in *Staphylococcus Aureus* Isolates from Raw Milk

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ABSTRACT

Background and Objective: Antibiotic-resistant *Staphylococcus aureus* strains have become a problem in treatment of infections caused by *S. aureus*. This study aimed to evaluate antibiotic resistance in *S. aureus* isolates from raw milk and detect *femA* gene in these isolates, as a confirmatory test for identification of *S. aureus* species.

Methods: This cross-sectional study was performed on 110 raw milk samples. After culture in Cooked Meat broth, presence of *S. aureus* in grown colonies was confirmed in accordance with Iranian National Standard, No. 1194. Antibiotic resistance was then evaluated according to guidelines recommended by the Clinical Laboratory Standards Institute. *FemA-specific* polymerase chain reaction was performed on antibiotic-resistant strains using specific primers and standard strains to differentiate *S. aureus* from other species.

Results: *S. aureus* were found in 43 (39.09%) of the 110 collected samples. Among these isolates, 79.07% and 76.75% were phenotypically resistant to penicillin and ceftazidime, respectively. In addition, the *femA* gene was detected in all isolates.

Conclusion: The results of this study show a high prevalence of resistance to penicillin and ceftazidime among *S. aureus* strains isolated from raw milk.

Keywords: *Staphylococcus aureus*, Antibiotic Resistance, Polymerase Chain Reaction.

INTRODUCTION

Nowadays, *Staphylococcus aureus* is considered as one of the most important pathogens that cause food poisoning in hundreds of thousands of people around the world every year (1). The members of this genus has more than 20 species that have different habitats including skin, skin glands and mucous membranes of animals that can be spread via animal products such as cheese, milk and meat. *S. aureus* food poisoning is among the most prevalent types of poisoning in most countries (2, 3). The variety of the bacteria habitats and imprudent and uncontrolled use of broad-spectrum antibiotics such as third generation cephalosporins, macrolides and fluoroquinolones are the contributing factors responsible for emergence of antibiotic (methicillin, vancomycin)-resistant *S. aureus* strains (4,5). *FemA* is a *S. aureus*-specific gene that encodes one of the several factors necessary for methicillin resistance. Therefore, this essential gene could be isolated from methicillin-resistant *S. aureus* strains (MRSA) (6, 7). The *femA* gene encodes a 48kDa broad-spectrum protein that is involved in metabolism, cell wall synthesis, growth and activity of bacteria in culture medium. Mutation or inactivation of *fem* genes (A, B, C) reduces resistance to methicillin (8, 9). Although presence of *MecA* chromosomal gene is required for synthesis of penicillin binding protein 2a (PBP2a), two groups of factors are involved in regulation of its expression. The first group includes products of genes such as *femA*. The second group consists of environmental conditions such as osmolarity of culture medium (such as NaCl), body temperature, and incubation time (10). Most *S. aureus* strains are heterogeneously resistant to several antibacterial agents such as beta-lactams, aminoglycosides, macrolides, clindamycin and tetracycline (6, 11). Therefore, this study aimed to evaluate antibiotic resistance of *S. aureus* isolates from raw milk and detect the *femA* gene for molecular identification of *S. aureus*.

MATERIAL AND METHODS

This descriptive cross-sectional study was performed on 110 raw milk samples collected from milk tankers and traditional cattle farms

in cities of Mamasani and Rostam from April to August 2012. The samples were poured into sterile containers and transferred to Foodstuff Laboratory of Mamasani and Rostam (Iran) at Faculty of Paramedical Sciences under cold condition. After homogenization of the samples with normal saline under sterile conditions and enrichment using Cooked Meat broth (PBL Co, UK, Cat No.D2013) with 7.5% NaCl, incubation was done at 37 °C for 48 hours. Then, the grown bacteria were sub-cultured on Baird-Parker agar with added egg yolk. Diagnostic and differential tests such as coagulase, DNase, mannitol, Voges-Proskauer, catalase, and Gram staining were carried out for all isolates obtained in accordance with the Iranian National Standard No. 1194. The isolates were stored in tryptic soy broth (TSB) containing 15% glycerol at -20 °C until molecular and antibiotic susceptibility testing. Antibiotic susceptibility of isolates was evaluated by disk diffusion method according to guidelines recommended by the Clinical and Laboratory Standards Institute (12). Fourteen antibiotics including penicillin (10 µg), chloramphenicol (30 µg), ceftazidime (30 µg), erythromycin (15 µg), vancomycin (30 µg) (12,13), gentamicin (10 µg), rifampin (30 µg), trimethoprim (5 µg), ciprofloxacin (5 µg), oxacillin (1 µg) (12,14), tetracycline (30 µg), cloxacillin (5 µg), ceftriaxone (30 µg) and cephalothin (30 µg) were purchased from Padtan Teb Co. for this purpose. The results were reported as susceptible, moderately susceptible and resistant after 24 hours of incubation at 37 °C (12). Standard *S. aureus* strain ATCC 29213 (purchased from Bank of Bacteriology of Tarbiat Modarres University, Tehran) was used for quality control of antibiotic discs.

S. aureus isolates cultured in BHI for 24 hours were used for DNA extraction. DNA was extracted using the phenol-chloroform method. Genomic DNA was electrophoresed on 1% agarose gel and concentration of DNA was measured at 260nm using transilluminator device to analyze the quality of the purified genome. The following species primers were used for amplification of the *femA* gene:

femA-F: 5'- AACCTAGGATTTGAACATACTGGA -3'
femA-R: 5'- GACGTTTACCTTCTTCAATCTT -3'

The primers were designed based on the genome of *S. aureus*, checked on the Blast

website, and then purchased from CinnaGen Co. *S. aureus* ATCC29213 and sterile distilled water were used as the positive and negative control, respectively. PCR reaction solution with final volume of 25 µl contained PCR Buffer 10X (5 µl), dNTP mixture (2 µl), MgCl₂ (1.5 µl), Taq DNA polymerase (1 µl), each of the primers (0.5 µl), DNA template (5 µl) and 9.5 µl sterile double-distilled water. Later, PCR was performed using a thermocycler (Mastercycler Gradient, Eppendorf, Germany) beginning with 5 minutes initial denaturation at 95 ° C, followed by 30 cycles of denaturation at 94 ° C for 1 minute, annealing at 60 ° C for 1 min, extension at 72 ° C for 1 minute, and final extension at 72 ° C for 10 minutes. The final PCR product was electrophoresed on 1.5% agarose gel and then analyzed under UV-light after staining with ethidium bromide. Statistical analysis of data was carried out in SPSS software version 18, using Fisher's exact

and Chi-square tests. P-values less than 0.05 were reported as statistically significant.

RESULTS

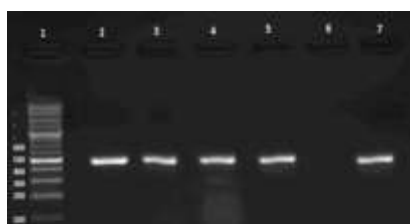
According to biochemical tests, 43 (39.09%) of the 110 samples collected were contaminated with *S. aureus*. The presence of the *femA* gene in all positive isolates were confirmed by the PCR experiment and observing a 499 bp band (Figure 1). Antibiotic susceptibility testing of *S. aureus* isolates showed that the highest level of resistance was to ceftazidime (79.07%) and penicillin (76.75%). On the other hand, the lowest level of resistance was observed against gentamicin (16.28%) and all isolates were susceptible to vancomycin. Simultaneous antibiotic resistance to multiple antibiotics including ceftazidime, penicillin, cloxacillin, oxacillin, trimethoprim, ceftriaxone, and cephalothin was observed in *femA* gene-containing strains (Table 1).

Table 1- Antibiotic susceptibility patterns of *S. aureus* isolates from raw milk samples using the disk diffusion method

	Susceptible (%)	Moderately susceptible (%)	Resistant (%)
Penicillin	8(18.60)	2(4.65)	33(76.75)
Chloramphenicol	28(65.12)	4(9.30)	11(28.58)
Ceftazidime	0(0)	9(20.93)	34(79.07)
Erythromycin	25(58.14)	10(23.26)	6(13.95)
Vancomycin	43(100)	0(0)	0(0)
Gentamicin	30(69.77)	6(13.95)	7(16.28)
Rifampin	27(62.79)	3(6.98)	13(30.23)
Trimethoprim	23(53.49)	7(16.28)	13(30.23)
Ciprofloxacin	21(48.84)	10(23.26)	12(27.9)
Oxacillin	23(53.49)	5(11.63)	15(34.88)
Tetracycline	26(60.47)	7(16.28)	10(23.26)
Cloxacillin	7(16.28)	11(25.58)	25(58.14)
Ceftriaxone	20(46.51)	14(32.56)	9(20.93)
Cephalothin	27(62.79)	7(16.28)	9(20.93)

Figure 1- Electrophoresis of PCR products

Column 1: 100 bp Marker bp, column 2: positive control (standard strain ATCC29213), column 3-5: *femA* gene 499 bp bands, column 6: negative control



DISCUSSION

S. aureus can colonize on skin and mucous membranes of humans and animals (as the primary reservoir). Thus, these bacteria can easily enter foodstuffs that require processing (meat, milk and vegetables) during preparation process. Most of the known species of these widely distributed bacteria in nature are saprophytes and nonpathogenic, but some of them are pathogenic for humans and animals. Presence of the bacteria in food and dairy products were investigated in the late nineteenth century (13). Bennet (1914)

showed the symptoms and effects of food poisoning by drinking milk contaminated with *Staphylococcus* (13). *S. aureus*, a common cause of food poisoning and infections caused by these bacteria, have a high prevalence in different places such as hospitals, which often have serious consequences despite antibiotic treatment. Therefore, determination of antibiotic resistance patterns of *S. aureus* isolates from different samples is essential, because it could be useful for selection of suitable drugs for treatment of the infections.

In this study, microbiological and biochemical methods showed that 43 (39.09%) of 110 milk samples collected were contaminated with *S. aureus*. The highest level of antibiotic resistance was observed against ceftazidime (79.07%) and penicillin (76.75%), while all isolates were susceptible to vancomycin. Increasing drug resistance in these bacteria and the consequent spread of infections, have drawn the attention of the scientific community. Study of Dibaj et al. investigated antibiotic susceptibility of 115 nasal *S. aureus* isolates from children in the city of Isfahan by the disc diffusion method, and found that all isolates were vancomycin-resistant (14), which is consistent with the results of the present study in regards to vancomycin resistance. Study of Farzana et al. was conducted on 50 raw milk samples collected from city of Multan in Pakistan, and reported that 40% of the samples were contaminated with *S. aureus* (15). Another study in Brazil on 162 raw milk samples collected from five cattle farms, reported that 70.4% of the samples were contaminated with *S. aureus* (16). In Nigeria, Suleiman et al. examined 339 milk samples obtained from animals with mastitis and reported that 103 samples (30.9%) were contaminated with *S. aureus*, while 35.6% of the isolates were oxacillin-resistant (17). These results are slightly different from the results obtained in the present study, which could be due to differences in climatic conditions and method of raw milk collection. Daka et al. found 78 *S. aureus* isolates in 160 raw milk samples collected in Ethiopia. All the isolates were resistant to penicillin G and the highest level of resistance was observed against amoxicillin (70.9%), penicillin (67.9%), oxacillin (60.3%) and vancomycin (38.5%) (18). Joshi et al. studied 400 milk samples and found 119 (29.7%) *S. aureus* isolates with 97.47%, 94.95%, 91.59% and 89.9% susceptibility to ciprofloxacin, gentamicin, ceftriaxone and tetracycline, respectively (19). These results are inconsistent with the findings of the present study, which could be related to the type of antibiotics used. Another study in Brazil, found 105 contaminations with *S. aureus* from a total of 465 milk samples. The highest rate of antibiotic resistance was observed against ceftazidime (n = 41, 89.1%) and cloxacillin (n = 41, 89.1%) (20). Poorfeiz et al. conducted a study in Tabriz on 220 raw

milk samples and reported 20 samples with *S. aureus* contamination. The percentage of antibiotic resistance to tetracycline, amoxicillin, gentamicin and erythromycin were reported as 35%, 5%, 45% and 40%, respectively. Moreover, all samples were sensitive to methicillin and vancomycin (21). These findings are consistent with the results obtained in the present study in terms of susceptibility to vancomycin, ceftazidime, cloxacillin and tetracycline. According to NCCLS recommendations, oxacillin could be used to determine susceptibility to methicillin. Oxacillin was used instead of methicillin in this study for identification of MRSA strains, since oxacillin maintains its activity for long-term and is more stable than methicillin in vitro (6,11,22). Thus, oxacillin is more efficient than methicillin in identification of heteroresistant strains (MRSA strains with lower numbers and growth rates in a microbial population compared to nonresistant strains). Although oxacillin resistance is rare in *S. aureus* strains, some of them could be resistant to methicillin or oxacillin and lack the gene coding for antibiotic resistance (12). The inconsistency in the frequency and extent of antimicrobial resistance reported by studies in Iran and rest of the world (15, 17-21) could be due to different of the techniques used, geographic area, and type of samples collected and common antibiotics prescribed during a particular period of time. High level of contamination in raw milk could have several reasons including cross-contamination caused by preparation by hand and failure to comply with code of hygienic practices for the milking process. Thus, presence of antibiotic-resistant strains in milk could pose a serious threat to public health. The percentages obtained for resistant strains are variable in different studies, which could be related to method of identification of the strains and type of samples studied. The PCR experiment in the present study showed that 43 isolates had the *femA* gene. Study of Janesh manikandan et al. in India detected the *femA* gene in all MRSA isolates (6). Li et al. found a similar finding in a study on MRSA isolates and reported increased expression of this gene in MRSA strains compared to methicillin-susceptible *S. aureus* (MSSA) strains (9). Kobayash et al. detected *femA* and *femB* gene in 237 *S. aureus* isolates and claimed that these two genes could be used for identification of MRSA strains (23). These

results are consistent with the results of the present study, which indicate the presence of this gene in all resistant and susceptible *S. aureus* strains. In fact, this *S. aureus*-specific gene is considered an essential factor for methicillin resistance.

CONCLUSION

There is a high prevalence of antibiotic resistance in *S. aureus* isolates from milk.

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CONFLICT OF INTEREST

We have no conflict of interest to declare.

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