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Short Communication

Restriction Fragment Length Polymorphism Typing of *Staphylococcus aureus* Strains Isolated from Bovine Mastitis and Dairy Products in Ahvaz, Iran, Using of Digested Coagulase Gene

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ABSTRACT

Staphylococcus aureus is a major pathogen in the transmission of diseases from animals to humans and vice-versa. Various infections, such as mastitis in cattle, sheep and goats, as well as gastroenteritis due to food poisoning in humans are the most frequent problems caused by *S. aureus*. The bacteria also lead to severe economic losses in dairy industry. A major virulence factor for the organism is encoded by the coagulase (*coa*) gene. This study aimed to assess the polymorphisms of the *coa* gene in *S. aureus* strains isolated from bovine mastitis and dairy product samples in Ahvaz, Iran. The results showed that out of 91 *S. aureus*, 80 (87.91%) isolates were positive for *coa* gene(s). In total, nine different polymerase chain reaction (PCR) products were obtained for coa-positive isolates. A single band was detected in *coa* PCR with a size ranges from 370 to 830 bp in most isolates (n=77, 96.25%). For three isolates (3.75%), two amplification products were obtained. A PCR product of an estimated size of 590 bp was most frequent, as obtained for 48 (60.00%) isolates. Whereas, 370 and 830 bp PCR products were the least presented, for two (2.50%) and one (1.25%) isolate, respectively. Subsequently, for restriction fragment length polymorphism (RFLP), typing of *coa* gene and AluI restriction enzyme were used for the digestion of the products. AluI for most of PCR products generated a unique pattern; however, four PCR products (the sizes ranged 750, 670, 590, and 510 bp) generated three or more patterns. Based on AluI RFLP of *coa* gene, the isolates were classified into 23 groups. Two groups of isolates were dominant, making 45% of the total. According to the findings, one or two types of *coa* RFLP were dominant among samples that were infected with more *S. aureus* isolates belonging to different coa RFLP types.

Keywords: *Staphylococcus aureus*, Coagulase Gene, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism , Bovine Mastitis, Dairy Products, Iran

Typage des souches de *Staphylococcus aureus* isolées à partir de mastites bovines et de produits laitiers à Ahvaz (Iran), en utilisant le polymorphisme de la longueur des fragments de restriction du gène de la coagulase

Résumé: Staphylococcus aureus est un agent pathogène majeur dans la transmission de maladies d'animaux à l'homme et vice versa. Diverses infections, telles que les mammites chez les bétails, moutons et chèvres, ainsi que les gastro-entérites dues à une intoxication alimentaire chez l'homme, sont les problèmes les plus fréquemment causés par *S. aureus*. La bactérie entraîne également de graves pertes économiques dans l'industrie laitière. L'un des facteurs de virulence majeur pour ces microorganismes est codé par le gène de la coagulase (*coa*). Cette étude visait à évaluer les polymorphismes du gène *coa* dans les souches de S. aureus isolées à partir d'échantillons de mammites bovines et de produits laitiers à Ahvaz, en Iran. Les résultats ont montré que 80

réaction en chaîne de la polymérase (PCR) ont été obtenus pour les isolats coa-positifs. Pour la plupart des isolats analysés, le produit de la PCR coa était une bande unique de 370 à 830 pb (n=77, 96,25%). Pour trois isolats (3,75%), deux produits d'amplification ont été obtenus. Un produit de PCR d'une taille estimée de 590 pb était le plus fréquent et a été obtenu pour 48 isolats (60,00%). Alors que les produits de PCR de 370 et 830 pb étaient les moins représentés, uniquement détectés dans deux (2,50%) et un (1,25%) isolats, respectivement. Ensuite, pour le polymorphisme de longueur des fragments de restriction (RFLP), le typage du gène coa et de l'enzyme de restriction AluI ont été utilisés pour la digestion des produits. Dans la plupart des produits de PCR, AluI a généré un modèle unique. Cependant, quatre produits de PCR (avec des tailles de 750, 670, 590 et 510 pb) ont généré trois ou plusieurs modèles. Sur la base de la RFLP AluI du gène coa, les isolats ont été classés en 23 groupes. Deux groupes d'isolats étaient dominants, représentant 45% du total. Selon les résultats, un ou deux types de RFLP coa étaient dominants dans les échantillons infectés par plusieurs isolats de *S. aureus* appartenant à différents types de RFLP *coa*.

Mots-clés: *Staphylococcus aureus*, gène de la coagulase, réaction en chaîne de la polymérase, polymorphisme de la longueur des fragments de restriction, mammite bovine, produits laitiers, Iran

INTRODUCTION

Staphylococcus aureus is an important pathogen causing a variety of diseases in animals and humans. Mastitis, dermatitis, udder impetigo, tick pyaemia, botryomycosis, bumble-foot, arthritis, abscess, and suppurative (pus-forming) infections are the most frequent problems caused by *S. aureus* in animals (Markey et al., 2013; Umesha and Manukumar, 2018). Staphylococcal mastitis has major impacts on both economy and animal welfare in the production of dairy ruminants, especially cows. *S. aureus* is associated with various forms of mastitis, among which subclinical mastitis is of particular importance, as it goes unnoticed and continues to affect livestock production (Suleiman et al., 2012). *S. aureus* is recognized as the main cause of food poisoning and gastroenteritis in humans worldwide. In fact, foodborne diseases are commonly caused by staphylococcal food poisoning (SFP) (Shin et al., 2016). SFP involves the consumption of dairy products, milk, and meat, particularly in traditional foods. *S. aureus* strains have been isolated frequently from SFP cases (Tsegmed et al., 2007; Hasman et al., 2010). Food poisoning due to *S. aureus* is related to the production of heat-stable enterotoxins in foodstuffs. The bacterium can shed into milk through udders infected with clinical or subclinical mastitis and environmental contamination during the handling and processing of milk and other dairy products (Morandi et al., 2010). In addition, *S. aureus* is a major nosocomial pathogenic agent, which causes pneumonia, phlebitis, meningitis, deep-seated infections (e.g., osteomyelitis and endocarditis), and skin infections, and soft tissue infections (e.g., furuncles, carbuncles, cellulitis, and abscesses) (Shopsin and Kreiswirth, 2001; Gould, 2009). Natural populations of *S. aureus* isolates have shown considerable genetic heterogeneity, associated with variable strains in certain domains that are responsible for the emergence of different epidemiologic profiles (Abdulghany and Khairy, 2014). Therefore, in epidemiological studies, several molecular methods have been used for the identification and comparison of *S. aureus* isolates, including phage typing, multilocus enzyme electrophoresis, plasmid profiling, pulsed field gel electrophoresis, random amplified polymorphic DNA, ribotyping, polymerase chain reaction (PCR) of coagulase (*coa*) and protein A (*spa*) genes, and PCR-restriction fragment length polymorphism (RFLP)-based typing of coa and *spa* genes (Zadoks et al., 2002; Rabello et al., 2007; Mehndiratta et al., 2009; Okolie et al., 2015). Among the mentioned techniques, *coa* gene typing is a simple

and efficient method, which shows high specificity in the identification and discrimination of coa-positive *S. aureus* isolates (Aarestrup et al., 1995; Rodrigues da Silva and da Silva, 2005; Momtaz et al., 2011; Dallal et al., 2016; Shin et al., 2016). In the analysis of DNA sequence in the 3′-end region of coa gene, heterogeneity was detected in the 81-bp tandem SSRs region that could differ both in the number of tandem repeats and the location of AluI restriction sites among different isolates encoding 27 amino acid sequences in the C-terminus. After the amplification of the coa gene that encompasses this region, fragments of different lengths could be obtained depending on the number of repeats, which could be differentiated with AluI restriction enzyme (Goh et al., 1992). With this background in mind, the current study presents a precise and simple *S. aureus* genotyping method, which can be applied in epidemiological studies and infection control programs (Himabindu et al., 2009; Saei and Ahmadi, 2012). The purpose of this study was to determine *S. aureus* subtypes, isolated from bovine mastitis and dairy product samples, using *coa* gene polymorphism profile in Ahvaz, Iran.

MATERIAL AND METHODS

Bacterial isolates. In total, 91 isolates of *S. aureus* were collected from different regions of Ahvaz, Khuzestan province, Iran. Among these, 58, 9, and 24, isolates were obtained from raw cow milk, traditional food products ,such as Sambooseh (n=7), and Falafel (n=2), and subclinical bovine mastitic milk samples, in different regions of Ahvaz, respectively.

Biochemical profile. All the isolates were subcultured three times at 37 °C in a Brain Heart Infusion Broth (Merck, Germany) for 24 h. They were examined twice and reevaluated if there were any discrepancies.

DNA extraction and coa PCR. To prepare DNAs, a genomic DNA purification kit (Fermentas, Germany) was used, as recommended by the manufacturer. The coa gene was amplified using primers previously described by Hookey et al. (1998). The primer sequences are presented in Table 1. The PCR reaction was carried out in a total volume of 25 µl, consisting of 1.50 µl of each primer (10 pmol), five µl of DNA, 0.50 µl of dNTP mixture (10 mM each), 1.50 µl of $MgCl_2$ (25 mM), 2.50 µl of PCR buffer (10X), and 0.50 µl of Taq DNA polymerase (1.25 units) (SinaClon Bioscience, Iran). For amplification, the following cycling parameters were applied: initial denaturation at 95 °C for five min, followed by denaturation for 45 sec at 95 °C, annealing for 25 sec at 57 °C, extension for 25 sec at 70 °C in 30 cycles, and a final five-min extension at 72 °C in a mastercycler gradient thermocycler (Eppendorf, Germany).

RFLP-PCR. Restriction analysis of PCR products was performed with AluI enzyme (Fermentas, USA), as described by the manufacturer: 10 µl of PCR products, one µl of rapid digest enzyme, three µl of rapid digest buffer (10X), and 16 µl of nuclease-free water at 37 °C for 20 min.

Agarose gel electrophoresis. For separating the PCR products and digested fragments, 1% and 2% agarose gel electrophoresis (Fermentas, Germany) were performed, respectively at 90 v for 30 min with 0.50 µg ml^{-1} of safe stain (SinaGen, Iran). DNA fragments were visualized under ultraviolet light (UVitec, UK), and a 100-bp marker (Fermentas, Germany) was used as the standard for determining the size of coa and AluI-generated coa fragments.

Specificity test. For determining the specificity of primer pair test, DNA of *S. aureus* COL strain and *S. epidermidis* ATCC 12228 were used.

Reproducibility test. Five random isolates were examined over five consecutive days to evaluate PCR reproducibility. The reproducibility of RFLP-PCR was evaluated twice by AluI digestion of four products.

Data analysis. For determining the size of RFLP and PCR fragments, a standard curve was plotted using the distance passed through the gel and by the fragments of defined length (molecular-weight size marker). PCR genotypes and RFLP patterns were assigned numerical

codes (Table 3). For statistical analysis, Chi square test was performed in SPSS software (version 19.0) (SPSS Inc., Chicago, USA).

Table 1. Primer sequences

Gene	Primer sequence	Size of PCR product (bp)
COA	COAG2: 5′-ATAGAGATGCTGGTACAGG-3′ COAG3: 5′-GCTTCCGATTGTTCGATGC-3′	370-830

RESULTS AND DISCUSSION

PCR amplification of *coa* gene with primers COAG2 and COAG3, showed that out of 91 *S. aureus,* 80 (87.91%) isolates were *coa*-positive (Table 2). Among them, 55 (94.82%) , 21 (87.50%) , 4 (57.14%), and 0 (0.00%) samples of raw cow milk, subclinical bovine mastitic milk, Sambooseh, and Falafel contained *coa*-positive *S. aureus* isolates, respectively. For most isolates (n=77, 96.25%), a single band was yielded in *coa* PCR with an approximate molecular size ranging from 370 to 830 bp. On the other hand, three isolates (3.75%) gave two amplification products (Figure 1). A PCR product of an estimated size of 590 bp was the most frequently detected for 48 (60.00%) isolates. Whereas, 370 and 830 bp products were the least frequent presented in two (2.50%) and one (1.25%) isolate, respectively (Table 2). The AluI RFLP patterns relative to the frequency of PCR products and genotypes are presented in Tables 2 and 3, respectively. Some PCR products generated a single pattern; however, four products (750, 670, 590, and 510 bp) generated three or more patterns. The agarose gel analysis of AluI RFLP patterns showed 23 different genotypes. Types 13, 14, 15, and 19 were the most frequent, accounting for 61.25% of the isolates. The 830, 750, 670, 670-750, 590, 590-510, 510, 510-670, and 370 bp amplicons generated different numbers of fragments, ranging from one to three (estimated size from 90 to 450 bp) (Figure 2). Moreover, 19 (90.47%) isolates from subclinical bovine mastitic milk samples contained one fragment (300 bp) and were classified as type 15; 2 (9.52%) other isolates after AluI digestion gave two fragments (450 and 220 bp) and were classified as type 12. In addition, two (50.00%) isolates from Sambooseh samples gave three fragments (220, 150, and 100 bp) and were classified as type nine; furthemore, two other isolates were classified as type 16 (Table 3). No amplification product was obtained from *S. epidermidis* DNA using the primers for *coa* gene. The PCR products were completely reproducible despite some variations in band intensity. The PCR fragments of two isolates were not digestible by AluI; therefore, this method showed a confidence of 97.50%. The product *coa* gene is a main virulence factor for *S. aureus* strains (Goh et al., 1992). It consists three different regions, such as N-terminus including the prothrombin-binding site, a highly conserved central region, and C-terminus, encoding 27 amino acid residues repeats (Janwithayanuchit et al., 2006). The 3′ end region of *coa* gene consists of tandem SSRs (81 bp).The variability of this region is the basis for the development of an easy and precise typing method of isolates from various sources (Goh et al., 1992; Aarestrup et al., 1995). Many different genotypes were detected among the studied isolates using this molecular method, which suggest that *S. aureus* has considerable heterogeneity in the sampled region. In the present investigation, *S. aureus* subtypes isolated from different sources in Ahvaz, Iran, were identified via PCR-based typing techniques (*coa* gene typing and PCR-RFLP). According to the current study, *coa* gene was detected in 87.91% of *S. aureus* isolates. Nine *coa* PCR types, ranging from 370 to 830 bp, were also detected, with one consisting of more than 59% of isolates (Table 2). Therefore, in the studied region, most cases of bovine mastitis and food samples may be caused by *S. aureus* strains with the same *coa* genotype. These findings are consistent with the observations of previous studies (Coelho et al., 2009; Saei et al., 2009; Khichar et al., 2014). Nevertheless, the other researchers have reported wider ranges of *coa* PCR products, such as 484 to 1080 bp (Da Silva et al., 2006), 500 to 1400 bp (Karahan and Cetinkaya, 2007), and 579 to 1442 bp (Rodrigues da Silva and da Silva, 2005). Overall, in 96.25% of the isolates, single-band

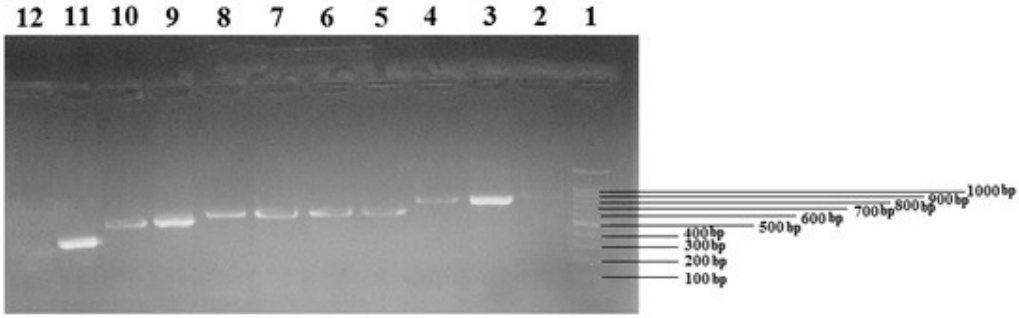

Figure 1. PCR products for coagulase (*coa*) gene of *S. aureus* representative isolates from bovine mastitis and dairy products samples on 1% agarose gel: Lane 1, 100-bp ladder; Lane 3, *S. aureus* COL strain, positive control; Lane 12, *S. epidermidis* ATCC 12228, negative control; Lanes 2 and 4-11, PCR products with different sizes

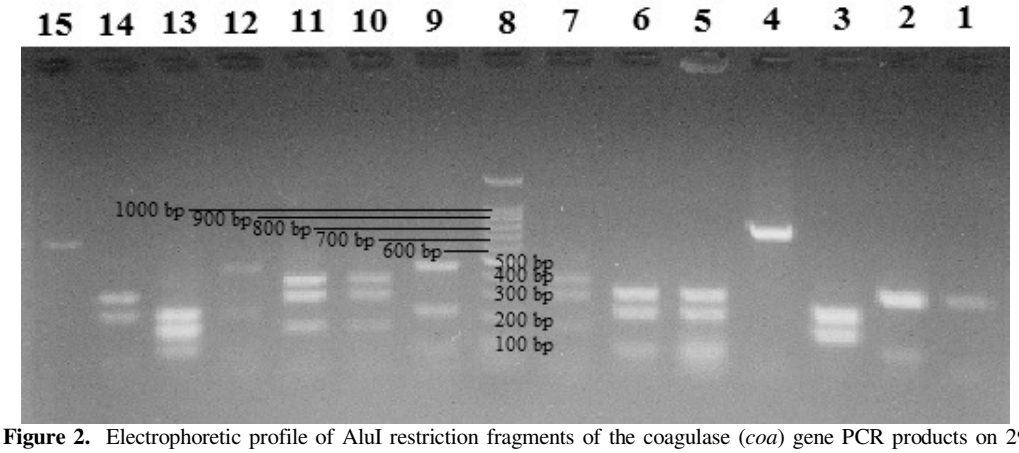

Figure 2. Electrophoretic profile of AluI restriction fragments of the coagulase (*coa*) gene PCR products on 2% agarose gel: Lane 8, 100-bp ladder; lanes 1–7 and 9-15, fragments with different patterns

Table 2. Numbers of distinct patterns of restriction fragment length polymorphism (RFLP) determined by *Alu*I testing of polymerase chain reaction (PCR) products of the coagulase (*coa*) gene of *S. aureus* isolated from different sources

PCR products (approximate size in base pairs (bp))	Number of isolates	Frequency (%)	Number of distinct RFLP patterns	Origin
830	1	1.25	1	RCM [a]
750	6	7.50	3	RCM
670	9	11.25	5	RCM= 7 S [b]= 2
670-750	1	1.25	1	RCM
590	48	60.00	7	RCM= 25 SBMM [c]= 21 S= 2
590-510	1	1.25	1	RCM
510	11	13.75	3	RCM
510-670	1	1.25	1	RCM
370	2	2.50	1	RCM
Total	80	100.00	23	-

[a] RCM= row cow's milk
[b] S= Sambooseh (traditional food product)
[c] SBMM= subclinical bovine mastitic milk

for *coa* PCR was observed, whereas in 3.75% of *coa*-positive isolates, double-band products were identified. In previous studies, *S. aureus* strains, which were isolated from bovine milk samples, gave single-band

amplification products (Raimundo et al., 1999). However, *S. aureus* strains from bovine mastitis have also given two PCR fragments for *coa* gene in several countries (Rodrigues da Silva and da Silva, 2005; Karahan and Cetinkaya, 2007). In this regard, (Goh et al. 1992) examined double-band products with various allelic *coa* gene forms (Goh et al., 1992). After the enzymatic digestion with AluI on *coa*-positive PCR products, 23 different genotypes were observed in the RFLP analysis. In other studies performed outside Iran, six to 49 AluI restriction patterns have been detected in bovine *S. aureus* isolates (Aarestrup et al., 1995; Raimundo et al., 1999; Rodrigues da Silva and da Silva, 2005; Karahan and Cetinkaya, 2007). Our findings showed that two types (13 and 15) are the predominant profiles among *S. aureus* strains in the study area. Overall, 19 strains (23.75%) related to genotype 15 contained one band of 300 bp, and 17 strains (21.25%) of genotype 13 consisted of three separate bands of 300, 200, and 90 bp (Table 3). These profiles seem to predominantly contribute to *S. aureus* pathogenicity in mastitis and food poisoning. In this

Table 3. Frequency of coagulase (*coa*) genotypes in the isolated *S. aureus*

Type code	Genotype		Number of isolates	Frequency (%)
	*PCR products (approximate size in base pairs (bp))	*RFLP pattern (approximate size in base pairs (bp))		
1	830	350-300-150	1	1.25
2	750[a]		1	1.25
3	750	350-300-150	3	3.75
4	750	450-220	2	2.50
5	670	350-300-150	1	1.25
6	670	300-200-90	1	1.25
7	670	300-200	4	5.00
8	670	300-90	1	1.25
9	670	220-150-100	2	2.50
10	670-750	670-300	1	1.25
11	590[a]		1	1.25
12	590	450-220	2	2.50
13	590	300-200-90	17	21.25
14	590	300-200	6	7.50
15	590	300	19	23.75
16	590	220-150-100	2	2.50
17	590	200	1	1.25
18	590-510	300-200-90	1	1.25
19	510	300-200-90	7	8.75
20	510	300-200	3	3.75
21	510	200	1	1.25
22	510-670	300-200	1	1.25
23	370	200-150	2	2.50
Total	-	-	80	100.00

[a] Not digested by *Alu*I
*PCR= polymerase chain reaction
*RFLP= restriction fragment length polymorphism

study, a remarkable heterogeneity was observed for *coa* gene of strains isolated in Ahvaz, Iran. Our results are in line with those obtained by the other authors. Saei et al. (2009) observed that isolates with similar and different *coa* products generated different and similar RFLP profiles, respectively. Nine PCR types and 23 AluI RFLP models showed that the isolated *S. aureus* strains harbored more than one variant of *coa* gene. Based on the findings, despite the presence of different genotypes, only one or two were predominant. These results are in line with the findings of previous research on bovine mastitis in different countries (Güler et al., 2005; Moon et al., 2007; Momtaz et al., 2011) and showed that a limited number of *coa* genotypes were predominant in each region and/or country. Güler et al. (2005) typed 125 *S. aureus* isolates from subclinical mastitis cow milk samples in Turkey and observed four PCR products of 1000, 900, 800, and 700 bp, with the 1000-bp product as the predominant (Güler et al., 2005). Moreover, Moon et al. (2007) investigated *S. aureus* isolates from different sources in Korea and identified 12 types, varying with the source of the organisms. Nevertheless, only a few genotypes were predominant in each source (Moon et al., 2007). Based on the findings reported by Hennekinne et al. (2012), SFP is one of the most common food-borne diseases caused by the ingestion of staphylococcal heat-stable enterotoxins produced in food by enterotoxigenic microorganisms (Hennekinne et al., 2012). In addition, Momtaz et al. (2011) typed 86 *S. aureus* isolates from samples of bovine clinical mastitis in two provinces of Iran and found five different genotypes. One predominant type was found among the isolates in the studied regions (Momtaz et al., 2011). Furthermore, in studies conducted by Su et al. (1999) and Mullarky et al. (2001), microorganisms with the dominant *coa* genotypes showed greater resistance to the host immune factors, such as neutrophil activity, compared to those with rare genotypes. Therefore, the dominant types might have particular characteristics, which can help to overcome the host defense mechanisms (Su et al., 1999; Mullarky et al., 2001). This study indicated that *coa* gene analysis could be beneficial for typing *S. aureus* strains isolated from different sources, such as bovine mastitis and dairy products. Furthermore, although the results showed that the samples in the studied region were infected with *S. aureus* strains harboring more than one *coa* genotype, only one or two genotypes were predominant. Further research is recommended to identify the features of dominant strains using greater number of strains. Such studies could help present control measures for staphylococcal mastitis and dairy food contamination by *S. aureus*.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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