

Genotypic Analysis of *Staphylococcus aureus* Coagulase Gene using PCR-RFLP analysis

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ABSTRACT

Background and Objectives: Determining the genetic relationship between *S. aureus* isolates is important for epidemiological surveillance and control of infections caused by this bacterium. The present study was conducted to determine polymorphisms of coagulase gene (*coa*) among *S. aureus* isolates from pastry and cheese samples using restriction fragment length polymorphism (RFLP) analysis.

Methods: Overall, 65 *S. aureus* isolated from pastry (n=45) and cheese (n=20) samples were examined for the *coa* gene by polymerase chain reaction (PCR). PCR products were digested with *AluI* enzyme and the products were assessed using gel electrophoresis.

Results: Except for two isolates, all isolates were positive in *coa*-PCR and produced four different PCR products, with molecular sizes ranging from 570 to 970 bp. Overall; five distinct RFLP patterns were detected (I-V). Although pattern types I and III were present in isolates from both samples, types I and IV were mainly present in isolates from cheese and pastry samples, respectively.

Conclusion: PCR-RFLP analysis of the *coa* gene indicates that *S. aureus* isolates from pastry and cheese samples may be originated from different sources. However, as one pattern type was predominant in each group, it can be concluded that majority of the isolates may have the same origin.

Keywords: *Staphylococcus aureus*, PCR-RFLP, Coagulase, Pastry, Cheese.

INTRODUCTION

Staphylococcus aureus is the second most important cause of foodborne diseases (1), which can be transmitted through milk and dairy products (2). Bacterial contamination in these products, directly from animals or from humans during production, may lead to food poisoning (3). For effective prevention and control of *S. aureus* infections, possible source(s) of infections should be identified by typing of isolates using different methods (4). In the past few years, various molecular techniques including pulsed field gel electrophoresis and multilocus sequence typing have been used for discrimination of *S. aureus* isolates (5,6). However, these methods are time consuming, not widely available and expensive (7). On the other hand, polymerase chain reaction (PCR)-based typing methods such as PCR-restriction fragment length polymorphism (PCR-RFLP) and random amplified polymorphic DNA-PCR are cheaper and have high discriminatory power and reproducibility (8,9).

Various polymorphic genes such as coagulase gene (*coa*) can be targeted for typing of *S. aureus* isolates. Pathogenic staphylococci produce coagulase, an extracellular virulence factor that can cause plasma coagulation. In fact, it acts as a cofactor and induces a conformational change in prothrombin, which converts fibrinogen to fibrin (10). Since this enzyme has polymorphic repeat regions in its 3' end sequence, it is thought that RFLP analysis of the *coa* gene could be useful for typing of *S. aureus* strains (11). Variable region of the *coa* gene is composed of 81 bp tandem short sequence repeats (SSRs) that encode tandem repeats of 27 amino acids in the C-terminal region. Difference in the number of SSRs and its mutations may cause distinct PCR amplification of this region and lead to generation of DNA fragments of different sizes, which could be discriminated further by digestion with restriction enzymes such as *AluI* (12). The present study was conducted to describe genotypic variations among *S. aureus* strains isolated from pastry and cheese samples.

MATERIAL AND METHODS

The study included 65 *S. aureus* strains isolated from pastry (n=45) and cheese (n=20) samples between November 2014 and April 2015. Identity of all isolates was confirmed by biochemical tests and species-specific PCR (data are not shown).

DNA was extracted from 10 ml overnight cultures of the isolates in tryptic soy broth (Merck, Germany) using phenol-chloroform method as described previously (13).

The DNA samples were amplified by PCR assay using *coa*-specific primers (Table 1) (14). The PCR reaction solution (50 µl) contained 5 µl of template DNA, 5 µl of 10× PCR buffer (500 mM, KCL and Tris HCL, pH 8.4), 1 µl (25 mM) of MgCl₂, 1 µl (200 mM) of dNTPs, 1 µl (50 pmol) of each primer (SinaClon, Iran), 35 µl of distilled water and 2 U Taq DNA polymerase (SinaClon, Iran). The amplification process was performed under the following conditions: initial denaturation at 94 °C for 5 min followed by denaturation at 94 °C for 60 sec, annealing at 58 °C for 90 sec and extension at 72 °C for 90 sec (32 cycles) and a final extension at 72 °C for 10 min (15). The PCR products were electrophoresed on 1% agarose gel containing ethidium bromide (0.5 µg/ml).

RFLP

The PCR products were purified using commercial gel purification kits (Vivantis, Malaysia), and digested with *AluI* enzyme (Fermentas, USA). Then, 10.5 µl of distilled water, 2.5 µl of 10× restriction buffers and 7 µl of the purified PCR products were mixed with 2 U of *AluI*. The mixture was incubated at 37 °C for 1 hour (14). The PCR products digested were electrophoresed on 2% agarose gel at 90 V for approximately 70 min. Finally, the patterns obtained from the RFLP assay were analyzed using GelCompar II software (version 6.5). The similarity index was calculated using the Dice coefficient with a band position tolerance of 1%. In addition, the unweighted-pair group method was used to construct a dendrogram, and clusters were selected using a cutoff of 40%.

Table 1- Primers used for amplification of the *coa* gene

Primer	Sequence 5'- 3'	Product size (bp)
COAG2-F	ATAGAGATGCTGGTACAGG	570-970
COAG3-R	GCTCCGATGTTCGATGC	

RESULTS

In PCR amplification of the *coa* gene, all isolates from cheese samples generated three DNA fragments ranged ~860 to ~970 bp (Figure 1). The most frequent (60%) PCR product among isolates from cheese was a 970-bp DNA band. On the other hand, 95.6% (43) of the *S. aureus* isolates from pastry samples contained the *coa* gene, and yielded 570, 860 and 970 bp DNA bands (Figure 1). However, two isolates (4.5%) did not show any band. Moreover, 74.4% of the isolates showed the 860 bp band. Later, RFLP was used to demonstrate polymorphisms of the *coa* gene among the *S. aureus* isolates. As shown in table 2, digestion of the PCR products with

AluI yielded different restriction patterns for isolates from pastry (4) and cheese (3) samples. The most frequent pattern in isolates from pastry was type IV, while type I was the most common pattern observed in isolates from cheese. Moreover, types I and III restriction patterns were common among the isolates from cheese and pastry (Figure 2). Based on the dendrogram generated, *S. aureus* isolates were divided into two major clusters (A and B) and four subclusters (Figure 2). All strains in subcluster A-1 were isolated from pastry samples. However, strains in cluster B and subcluster A-2 were isolated from pastry and cheese samples.

Figure 1- Amplification of the *coa* gene in *S. aureus* isolates from pastry (A) and cheese (B) samples. L: 100 bp DNA ladder, column 1: positive control (*S. aureus* ATCC 25923), column 2: negative control (no template DNA), columns 3-5: *S. aureus* isolates

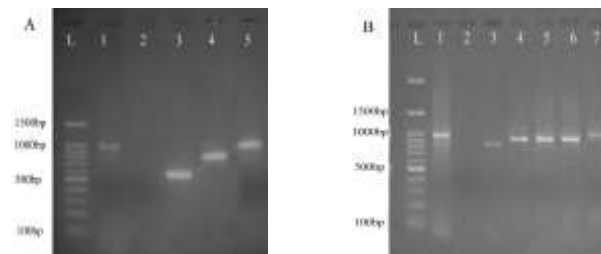


Table 2- Types of *S. aureus* isolates based on the RFLP patterns for the *coa* gene

Type	PCR product (bp)	<i>AluI</i> profiles	Isolates cheese	from	Isolates from pastry
-	No bond	-	0		2
I	970	160-320-490	12		6
II	920	160-760	2		0
III	860	160-700	6		2
IV	860	160-280-410	0		32
V	570	160-410	0		3
Total			20		45

Figure 2- RFLP patterns obtained from digestion of PCR products. Column 1: type IV, column 2: type I, column 3: type V, column 4: type III, column 5: type II

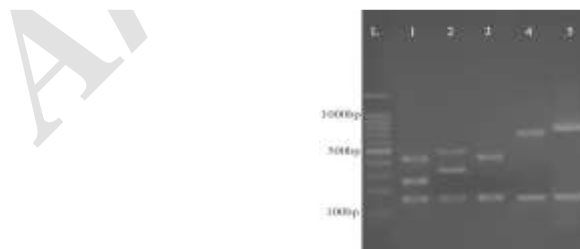
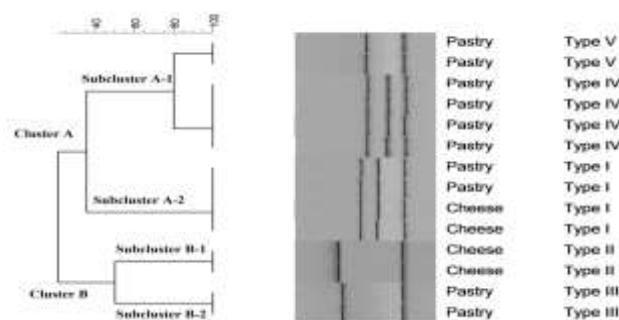


Figure 3- Dendrogram of genetic relationship among various RFLP types in *S. aureus* isolates



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DISCUSSION

Coagulase is one of the virulence factors of *S. aureus*, which may contribute to its pathogenicity (16). The enzyme is encoded by the *coa* gene, which is highly polymorphic at its 3' end. PCR amplification of this gene may yield amplicons of different sizes, which could be useful for differentiation of *S. aureus* isolates and determination of the possible source of infection.

In the present study, PCR amplification of the *coa* gene revealed significant differences between *S. aureus* isolates from pastry and cheese samples. Generally, all isolates from cheese were *coa*-positive and only two isolates (4.44%) from pastry were *coa*-negative. In accordance with these results, some studies have previously shown that *S. aureus* isolates may be negative for the *coa* gene. Momtaz et al. reported that only 42 of 86 *S. aureus* isolates in milk samples from bovine mastitis cases contained the *coa* gene in PCR-RFLP analysis (17). In study of Karahan and Cetinkaya, the *coa* gene was detected in 80.5% of *S. aureus* strains isolated from subclinical cases of mastitis (14). However, using the same *coa*-specific primers, the percentage of *coa*-positive isolates was much higher (97%) in our study. Nevertheless, findings of some studies were consistent with our findings (18, 19).

We found that 32 of 45 isolates from pastry showed an 860 bp DNA fragment in PCR. However, the majority of isolates from cheese showed a 970 bp DNA fragment related to the *coa* gene. Generally, the size of the *coa* gene in isolates from cheese was larger than that in isolates from pastry. In line with our results regarding isolates from pastry, some studies have reported that the *coa* gene PCR products from *S. aureus* isolates of human origin could be 650-900 bp long (20).

Tiwari et al. analyzed the *coa* gene in clinical and nasal *S. aureus* isolates using the same primers used in our study. They reported that the size of the gene might be 300-800 bp, and the 600 bp as the most common DNA fragment (19). In a study by Reinosoa et al. in Argentina, PCR amplification of the *coa* gene in *S. aureus* isolates from human, bovine and food samples yielded seven different *coa* types with amplicon sizes between 400 and 1000 bp (21). In study of Scherrer et al., 285 (97.3%) of 293 *S. aureus* isolated from raw milk samples showed a single DNA band (500-820

bp) in PCR of the *coa* gene. In RFLP analysis of PCR products with *AluI*, they reported six different restriction patterns (A-F) with pattern B (80-180-210) as the most common type. In addition, they reported different *coa*-RFLP patterns among *S. aureus* isolates from goat, sheep and bovine milk samples (22). Momtaz et al. reported the presence of certain *coa* genotypes of *S. aureus* in cases of mastitic in dairy herds in Isfahan, Iran (17). Talebi-Satlou et al. reported tissue-specific tendency in RFLP analysis of the *coa* gene variants among 26 *S. aureus* isolates from different human tissues (23). Omar et al. reported that *coa*-RFLP analysis of *S. aureus* isolates was more useful for local epidemiologic purposes (24).

In the present study, all PCR products were digested with *AluI* and five distinct (I-V) RFLP patterns were detected. Little similarity was found between isolates from pastry and cheese samples. Pattern types IV and V were only detected in isolates from pastry, while pattern type II was only detected in isolates from cheese. However, at 35% similarity, the dendrogram showed that *S. aureus* isolates from pastry samples were only slightly related to the isolates from cheese samples. These results indicate that these isolates may be of different origin (humans or animals). In this regard, we have previously found that most human- and bovine-associated *S. aureus* isolates produce 860 bp and 970 bp DNA fragments in *coa*-PCR (data have not yet been published), respectively. Pastry isolates may originate from humans, whereas cheese isolates could belong to bovine milk. However, some isolates from both samples shared the same RFLP patterns (types I and III). This indicates that such strains may circulate among different sources or hosts. Consistent with our findings, some studies suggested transmission of *S. aureus* between animals, humans and food samples (25, 26).

CONCLUSION

The present study demonstrated that *S. aureus* isolates from pastry and cheese samples are genetically diverse, and can be typed by *coa*-PCR-RFLP analysis. According to the results of RFLP, there is only a slight similarity between pattern of isolates from pastry and cheese samples, suggesting that these isolates may be of different origin. However, one

pattern type was predominant among the isolates from both samples, which could indicate their common origin of infection.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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