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Original Article

Detection of Classic Enterotoxin Genes and Coagulase Gene Typing of *Staphylococcus Aureus* Isolated from Raw Cow Milk in Isfahan

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Background and Aims: *Staphylococcus aureus (S. aureus)* is considered as one of the most dangerous pathogenic bacteria due to the production of extracellular toxins. The objective of this study was to determine the prevalence of *S. aureus* and to characterize the recovered strains for their enterotoxin-producing genes in raw cow milk.

Materials and Methods: During 9 months duration of the study, a total of 322 raw milk samples were collected from different markets in Isfahan province in Iran. *S. aureus* isolates were identified by bacteriology and biochemical tests. The isolates were typed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for detection of *coa* gene and genes encoding classic enterotoxins (*sea*, *seb*, *sec* and *sed*).

Results: A total of 109 strains were positive for at least one type of staphylococcal enterotoxin genes with *sea* being the predominant. The isolates were grouped into 3 genotypes I, VIII and IX using RFLP analysis results of the genes.

Conclusions: The alarmingly high prevalence of *S. aureus* and their enterotoxin genes in raw cow milk should raise awareness about the food safety of such milk and milk products.

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Introduction

Staphylococcus aureus (S. aureus) is the causative agent of many opportunistic infections in human and animals [1]. Among animals, cows whose milk is frequently used, can act as the leading cause of intramammary infections, with major economic repercussions [2, 3]. An outbreak on a farm is often caused by a single strain and may lead to further outbreaks among the same species in the same region.

Milk and milk products have frequently been implicated in staphylococcal food poisoning, and contaminated raw milk is often involved [4, 5]. *S. aureus* mastitis is a serious problem in dairy production, and infected animals may contaminate bulk milk. Human handlers, milking equipment, the environment, and the udder and teat skin of dairy animals are other possible sources of bulk milk contamination [6, 7]. *S. aureus* is known to produce a variety of virulence factors such as the staphylococcal enterotoxins (SEs), exfoliative toxins and toxic shock syndrome toxin (TSST) which are responsible for specific acute staphylococcal toxaemia syndromes including staphylococcal food poisoning and scalded skin syndrome [8, 9]. Both SEs and TSST are members of the superantigenic toxin family that stimulate nonspecific T-cell proliferation [10-12]. Enterotoxins produced by the bacteria are believed to be wholly responsible for the symptoms of food poisoning [13]. Therefore, only enterotoxigenic strains of *S. aureus* are thought to be able to cause food poisoning. A total of 18 different types of enterotoxins such as SEA-SED, SEE, SEG-SER and SEU encoded respectively by *sea-sed, see, seg-ser* and *seu* genes, have been reported [12]. Among them, SEA is the most common enterotoxin found in food and is frequently associated with staphylococcal food-poisoning outbreaks worldwide [14].

Coagulase gene (*coa*) typing is a simple, accurate, reproducible enough, easy to interpret and discriminatory method for typing *S. aureus* isolates from various sources [15-18]. Prevalence and etiology of subclinical mastitis in dairy shows that coagulase-negative staphylococci are the most prevalent, ranging from 25% to 93% (mean value approximately 78%) of bacterial isolates. Of these staphylococci, *S. aureus* prevalence ranges from 3% to 37% (mean value approximately 4%) of the bacterial isolates [19]. Although many putative virulence factors have been identified in the *S. aureus* genome [20], the differences in pathogenicity between field isolates remain largely unknown [21].

To implement better control of subclinical mastitis in cows, and particularly *S. aureus*-induced mastitis, it is important to clarify the epidemiology of this major pathogen in raw cow milk. Little information is available regarding the molecular epidemiology of *S. aureus* and enterotoxin-producing gene as well as coagulase producing gene in raw cow milk. The aim of this study was to determine the prevalence of *S. aureus* isolates, and enterotoxin and coagulase producing genes of *S. aureus* recovered from raw cow milk samples.

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Material and Methods

Samples and identification of *S. aureus*

From February 2016 to October 2017, a total of 232 raw cow milk samples were collected from different markets in Isfahan province in Iran. All of the raw cow milk samples were immediately transferred to the laboratory in cool packs. For *S. aureus* enumeration [22], 1 ml of each milk sample was inoculated on Baird Parker agar (Merck, Germany) with 5% egg yolk tellurite emulsion (Liofilchem, Italy) and incubated at 35˚C for 48 h. Characteristic colonies were tested for catalase, coagulase production and mannitol fermentation. The two species positive for clumping were submitted to the Voges-Proskauer test to discriminate *S. aureus* (positive) from *S. intermedius* (negative). The strains were further identified as *Staph. aureus* by polymerase chain reaction (PCR) amplification of the *23S rDNA* according to Straub et al. [23].

DNA Extraction

Isolates were grown on blood agar (Merck, Germany) for 24 h, then a single colony was picked, resuspended in 100 ml of sterile deionized water, and heated at 99°C for 15 min. with mild shaking in a thermomixer comfort (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The tubes were then centrifuged at 1,000×*g* for 5 min. at 14°C to remove the sediment and supernatant containing crude extract of bacterial DNA was transferred into a new tube and kept frozen until being used for PCR amplification [24].

Detection of enterotoxin genes by PCR

All oligonucleotide primers used in this study were selected from earlier reports, as mentioned in table 1. The presence of the *sea*, *seb*, *sec*, and *sed* genes was examined using multiplex PCR assay introduced by Rosec and Gigaud [25].

Coagulase gene typing

PCR was performed in a 50-µl reaction mixture containing 2 µl of template DNA (approximately 500 ng/µl), 5 µl of 10×PCR buffer (750 mM Tris-HCl (pH 8.8), 200 mM (NH4) 2SO4, and 0.1% Tween 20), 200 µM of each of the four deoxynucleotide triphosphates, 1 U of Taq DNA polymerase (Fermentas), and 50 pmol of each primer (Table 2). An aliquot of 10 µl amplification product was digested with 2U of restriction endonuclease *Alu*I (Fermentas) at 37˚C for 4 h. According to the kit, the resulted fragments were migrated on 2% agarose gels, which were stained with DNA Safe Stain (CinnaGen, Iran), and the image was observed under ultra violet light. The software was used for the size of PCR and restriction fragment length polymorphism (RFLP) products. Numeric codes were assigned to the PCR genotypes and RFLP patterns [26]. This study was approved by the Ethics Committee of Islamic Azad University, Shahrekord, Iran.

Results

In the present study, the prevalence of *S. aureus* in raw cow milk samples showed to be 109/232 (46.98%). A total of 109 *S. aureus* isolates were screened for the expression of enterotoxin. The prevalence of enterotoxin-producing gene in the 109 *S. aureus* isolates from raw cow milk for 4 tested toxin genes were *sea* (85.32%), *seb* (62.38%), *sec* (14.67%) and *sed* (11.00%). In this study,

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three *coa* gene RFLP patterns, numbered I, VIII and IX, were observed, with 71 isolates (65.13%) assigned to RFLP pattern I, 29 isolates (26.60%) assigned to RFLP pattern VIII and 9 isolates (8.25%) assigned to RFLP pattern IX (Table 3). Amplification of the variable region of the *coa* gene from these isolates produced five different PCR products ranging in size from approximately 160 bp to 490 bp.

Table 1. Oligonucleotide primers and amplification conditions for detection of enterotoxin genes of *S. aureus* isolated from raw cow milk

Gene	Oligonucleotide sequence (5′-3′)	Product size (bp)
sea	Forward: ACGATCAATTTTTACAGC Reverse: TGCATGTTTTCAGAGTTAATC	544
seb	Forward: GAATGATATTAATTCGCATC Reverse: TCTTTGTCGTAAGATAAACTTC	416
sec	Forward: GACATAAAAGCTAGGAATTT Reverse: AAATCGGATTAACATTATCCA	257
sed	Forward: TTACTAGTTTGGTAATATCTCCTT Reverse: CCACCATAACAATTAATGC	334

Table 2. Oligonucleotide primers and amplification conditions for detection of Coagulase gene of *S. aureus* isolated from raw cow milk

Gene	Oligonucleotide sequence (5′-3′)	PCR program	Product size (bp)
COA	COAG2: CGA GAC CAA GAT TCA ACA AG COAG3: AAA GAA AAC CAC TCA CAT CA	Initial denaturation at 95°C for 2 min., 30 cycles of 30 s each with denaturation at 95°C, 2 min. annealing at 58°C, 4 min. extension at 72°C, and a final 7 min. extension at 72°C	730-1050

Table 3. Coagulase gene typing of *S. aureus* isolated from raw cow milk

The number of isolated	The pattern of bands (bp)	Genotype
71	490- 320- 160	I
29	290- 240	VIII
9	410- 320	IX

Discussion

S. aureus is known to be responsible for a variety of toxins-mediated diseases [11]. Although several studies have reported the genotypic characteristics and distribution of *S. aureus* in dairy herds in Iran [27], reports regarding the prevalence of selected virulence genes in *S. aureus* in raw cow milk in Iran are scanty. This report demonstrates detailed prevalence of enterotoxin and coagulase virulence genes of *S. aureus* isolated in raw

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cow milk in Iran. The contamination level of food contact surfaces with *S. aureus* suggests that the handling of raw cow milk and milk products must be improved.

The pathogenicity of food-borne *S. aureus* is associated with the ability of some strains to produce enterotoxins [28]. Several studies conducted on toxin genes and other characteristics of *S. aureus* isolates from milk of cows with mastitis revealed that the coexistence of *sec* and *tst*, and the coproduction of SEC and TSST-1, is frequently observed in *S. aureus* isolates from cases of ruminant mastitis [29-31] and probably reflects the colocation of these genes on the bovine *S. aureus* pathogenicity island [13]. Another study conducted in Tulsa, Oklahoma showed that the prevalence of toxin genes in the 168 *S. aureus* isolates from poultry were *sea* (1.2%), *seb-sec* (1.2%), *sec* (0.6%), *and sed* (0%) [32]. Reports from another study indicated that there is high prevalence of enterotoxin genes such as *seg*, *seh*, and *sei* and the toxic shock syndrome gene *tst*. The frequent presence of potentially SE producing *S. aureus* strains in raw milk and raw-milk products is a concern, since these may pose a public-health risk to consumers [33]. This increase might be caused by horizontal gene transfer among the strains as SEs genes are carried by mobile genetic elements such as plasmids, pathogenicity islands, *SCCmec* and prophages [34]. This is of public health concern as *SEs* genes are often associated with food-borne poisoning, toxic-shock syndrome and other toxin mediated disease [11, 12].

In this study, *sea* gene was the most common SEs gene present among Iranian *S. aureus* strains and this concurred with the finding reported from another tertiary hospital in Kuala Lumpur [15]. However, this differed from a report by Sauer et al. (2008) where *seg* and *sej* genes were predominant in the methicillin-resistant *S. aureus* (MRSA) strains in a University Hospital of Czech Republic [35]. Moreover, another study indicated that classical staphylococcal enterotoxins (SEA to SEE) have been reported to cause 95% of staphylococcal food poisoning and, SEA is the most common in staphylococcus-related food poisoning [36]. Another study conducted to characterize *S. aureus* and MRSA isolated from Louisiana retail pork and beef meats for the possession of toxin genes showed that the most prevalent ones were *seg* and *sei* followed by *seh*, *sed*, *sej*, and *sea* while no isolates harbored *seb*, *sec* or *see* [37]. In addition, a study performed in Italy reported that the prevalence of enterotoxin genes for *S. aureus* was 58.8% in meat and dairy products [38]. The difference prevalence of *S. aureus* and virulence genes among different studies could be due to sample type and sampling, processing facility and geographic location.

A study carried out on molecular typing of *S.aureus* isolated from cows, goats and sheep with intramammary infections revealed seven different coa types and 12 different spa types. On the basis of PCR-RFLP, 29 different *coa* subtypes were identified. Two different *coa* subtypes accounted for 49% and 67% of bovine and ovine isolates respectively. Only seven *coa* subtypes were observed in isolates from more than one host species and no *coa*

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subtype was present in isolates from all three ruminant species [39]. Another study conducted in Iran on molecular typing of *S.aureus* isolated from bovine mastitis showed that nine coa gene RFLP patterns, numbered I-IX, were observed, with 23 isolates (39.66%) assigned to RFLP pattern I and 14 isolates (24.14%) assigned to RFLP pattern III. Five out of nine patterns were found in both regions and four out of nine patterns were only found in one region [27]. This result is consistent with our finding and in general, the results demonstrated that several variants of the *coa* gene are present in the studied regions; however, only a few of them were predominant, suggesting contagious transmission, a common source, or host adaptation of subset of the population of *S. aureus* strains. This study also indicated that genetic heterogeneity among *S. aureus* isolates recovered from bovine mastitis may exist in raw cow milk in different regions.

Conclusions

S. aureus was highly prevalent and some of the recovered isolates possessed several toxin genes that are known to contribute to the virulence of this important food-borne bacterium. This finding strongly suggests that certain *S. aureus* strains predominant in the raw cow milk are perhaps more prone to adhere to and colonize cow udder because of the presence of particular virulence factors increasing their potential for adherence and colonization. The existence of a predominant strain of *S. aureus* suggests that this clone may have special properties to overcome host defense mechanisms and to establish a successful intra-mammary infection in cows. In this study, *S.aureus* strains from raw cow milk showed the highest incidence of the *sea* gene. Collaboration between veterinary and human healthcare workers will be necessary to help control the spread of this pathogen between humans and food production animals. The data from this research may act as reference for monitoring the prevalence of virulence genes among Iranian *S. aureus* strains. These results, therefore, point toward the need to improve hygiene conditions during milking and care should be taken not to use raw cow milk and not to leave cow milk for longer periods of time at room temperature prior to boiling to reduce the chance of thermostable enterotoxin production.

Conflict of Interest

All the authors declare to have no actual or potential conflict of interest

Acknowledgement

There is no acknowledgement to declare.

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