Short Paper

Enterotoxin gene profiles among *Staphylococcus aureus* isolated from raw milk

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Summary

Milk is considered a nutritious food because it contains several important nutrients including proteins and vitamins. Conversely, it can be a vehicle for several pathogenic bacteria such as *Staphylococcus aureus*. This study aimed to analyze the frequency of genes encoding the nine Staphylococcal enterotoxins (SEs) and enterotoxin gene profiles in *S. aureus* isolates derived from raw bovine milk. A total of 52 *S. aureus* isolates were obtained from 246 milk samples of 246 dairy cows from eight different farms in Qom, Iran. On the basis of cultural and biochemical properties as well as by amplification of the 23S rRNA specific to *S. aureus*, all isolates could be identified as *S. aureus*. Of the 52 isolates studied, 80.7% were positive for one or more genes encoding the enterotoxins, and 12 different genotypes were identified. The gene encoding for enterotoxin A (*Sea*) was the most frequent (16 isolates, 30.7%), followed by *Seb* (14 isolates, 26.9%) and *Sed* (8 isolates, 15.37%). Among the genes encoding the other enterotoxins, *Seg* and *Seh* were the most frequently observed (8 isolates each, 15.38%), followed by *Sej* (6 isolates, 11.5%) and *Sei* (1 isolates, 3.84%). With the recent identification of new SEs, the frequency of enterotoxigenic strains has increased, suggesting that the pathogenic potential of Staphylococci may be higher than previously thought. These results of enterotoxin genes positivity of milk-derived Staphylococci constitute a potential risk for consumers' health.

Key words: *Staphylococcus aureus*, Staphylococcal enterotoxins, Raw milk, PCR

Introduction

Staphylococcus aureus is a gram-positive bacterium, which produces many important virulence factors including Staphylococcal enterotoxins (SEs) which are responsible for Staphylococcal food poisoning (SFP), a major type of foodborne illness (Balaban and Rasooly, 2000). Classical SEs have been divided into five serological types (SEA through SEE) on the basis of their antigenicities. In recent years, the existence of new types of SEs (SEG-SEU, except SES and SET) have been reported (Ren *et al*., 1994; Su *et al*., 1995; Munson *et al*., 1998; Zhang *et al*., 1998; Jarraud *et al*., 2001; Orwin *et al*., 2001). Staphylococcal enterotoxins are resistant to inactivation by gastrointestinal proteases such as pepsin. In addition, they displayed strong thermo resistance, for example, SEA retains some biological activity after 28 min at 121°C (Anderson *et al*., 1996). Staphylococcal enterotoxins can be routinely detected by immunoassay, e.g. enzyme linked immunosorbent assay (ELISA), immunodiffusion, radioimmuno-assay and latex agglutination but the availability of these methods are usually limited to commercial tests for classical SEs (Omoe *et al*., 2002). Therefore, the DNA-based approach (PCR assays) is thought to be an essential tool for investigating SE genes (Omoe *et al*., 2002). The identification of *S. aureus* at the species level is based on amplification of target genes highly conserved within the species. In addition to the 16S rRNA gene, the well established standard target for the identification of bacterial species (Amann *et al*., 1995), the 23S rRNA genes has proven useful for identification of *S. aureus* at the species level (Akineden *et al*., 2001; Phuektes *et al*., 2003). The aim of this study was to identify *S. aureus* strains via amplification of the genes encoding the 23S rRNA and to analyze the genes encoding the Staphylococcal enterotoxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ in *S. aureus* strains isolated from raw bovine milk.

Materials and Methods

Sample collection and identification

A total of 52 *S. aureus* isolates were collected from milk sample of 246 cows from eight different farms in Qom, Iran. The identification of the isolates was performed by gram staining, catalase, hemolysis and tube coagulase test (Liofilchem, Italy) and was confirmed by PCR amplification of species specific parts of the *S. aureus* 23S rRNA gene.

Genotypic characterization

The DNA of isolates was prepared with the high pure

PCR template preparation kit (Roche, Germany) as described by the manufacturer. The sequences of the oligonucleotide primers, the predicted PCR product sizes and the references are summarized in Table 1. For PCR amplification, the reaction mixture (25 µl) contained 15 ng DNA template, 1 µl of primer F (10 pmol), 1 µl of primer R (10 pmol), 2.5 µl 10 X PCR buffer (Fermentas, Lithuania), 0.5 µl dNTP (10 mM, Fermantas, Lithuania), 1.5 µl $Mgcl_2$ (25 mM, Fermentas, Lithuania), 0.5 U Taq DNA polymerase (Fermentas, Lithuania) and double-distilled water to the final volume of 25 µl. DNA amplification was performed in a thermal cycler (Eppendorf, Germany) with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing 58°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The amplified PCR products were electrophoresed in a 1% agarose gel (Fermentas, Lithuania) containing ethidium bromide (Fermentas, Lithuania) and visualized by trans illumination under UV. Molecular size marker (Vivantis, Malaysia) was included in each agarose gel. The *S. aureus* reference strains ATCC 19095, ATCC 23235, ATCC 14458, ATCC 700699, ATCC 27664, ATCC 25923 were used as positive controls. The reference strains *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus saprophyticus* ATCC 14448 were used as negative controls.

Results

Staphylococcus aureus was observed in 52 (21.1%) samples of 246 raw milk samples. According to the results of PCR assay by amplification of the 23S rRNA gene specific to *S. aureus*, all 52 isolates contained 1250 bp DNA fragments bands and showed positive PCR assay. Table 2 shows the results of molecular tests for the detection of gene encoding the enterotoxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ. Of the 52 isolates of *S. aureus* tested, 42 (80.7%) were positive for one or more SE genes, and 12 different genotypes were observed. Among the 52 *S. aureus* isolates, 28 isolates (53.8%) harbored only one enterotoxin gene, 8 isolates (15.38%) carried gene coding for two enterotoxin. Genotypes encoding three enterotoxins (*Sea*+*Seb*+*Seh*+, *Sea*+*Seb*+*Sej*+, *Seb*+*Sed*+*Seg*+) were detected in 6 isolates (11.53%). Genes encoding the enterotoxins SEC and SEE were not observed separately. Among the genes that code for classic enterotoxins (SEA-SEE), *Sea* was the most frequent, it was found in 16 isolates (30.7%) followed by *Seb* in 14 (26.9%) and *Sed* in 8 (15.37%) isolates. Regarding the other enterotoxins, *Seg* and *Seh* were the most frequently observed (8 isolates each, 15.38%), followed by *Sej* in 6 (11.5%) isolates and *Sei* in 1 (3.84%) isolate (Fig. 1).

Table 2: Genotypic profile of *Staphylococcus aureus* strains isolated from raw milk according to SE genes

Genotypic profile	Number of strains
Sea	10
Seb	4
Sed	4
Seg	4
Seh	4
Sei	2
Sea+Sed	2
Seb+Sej	4
Seg+Seh	2
Sea+Seb+Seh	2
Sea+Seb+Sej	2
Seb+Sed+Seg	2

Table 1: Primers used for the detection of *Staphylococcus aureus* SE genes

Gene	Primer	Sequence (5´-3´)	Size (bp) of PCR product	Reference
23S rRNA	23S rRNA F	ACG GAG TTA CAA AGG ACG AC	1250	Straub *et al*. 1999
	23S rRNA R	AGC TCA GCC TTA ACG AGT AC		
Sea	sea F	TAA GGA GGT GGT GCC TAT GG	180	Cremonesi *et al*. 2005
	sea R	CAT CGA AAC CAG CCA AAG TT		
Seb	Seb F	TCG CAT CAA ACT GAC AAA CG	478	Johnson *et al*. 1991
	Seb R	GCA GGT ACT CTA TAA GTG CC		
Sec	sec F	ACC AGA CCC TAT GCC AGA TG	371	Cremonesi *et al*. 2005
	sec R	TCC CAT TAT CAA AGT GGT TTC C		
Sed	sed F	TCA ATT CAA AAG AAA TGG CTC A	339	Cremonesi *et al*. 2005
	sed R	TTT TTC CGC GCT GTA TTT TT		
See	see F	AGG TTT TTT CAC AGG TCA TCC	209	Mehrotra *et al*. 2000
	See R	CTT TTT TTT CTT CGG TCA ATC		
Seg	seg F	CCA CCT GTT GAA GGA AGA G	432	Cremonesi *et al*. 2005
	seg R	TGC AGA ACC ATC AAA CTC GT		
Seh	seh F	TCA CAT CAT ATG CGA AAG CAG	463	Cremonesi *et al*. 2005
	seh R	TCG GAC AAT ATT TTT CTG ATC TTT		
Sei	sei F	GGT GAT ATT GGT GTA GGT AAC	454	Omoe *et al*. 2002
	sei R	ATC CAT ATT CTT TGC CTT TAC CAG		
Sej	sej F	GGT TTT CAA TGT TCT GGT GGT	306	Cremonesi *et al*. 2005
	sej R	AAC CAA CGG TTC TTT TGA GG		

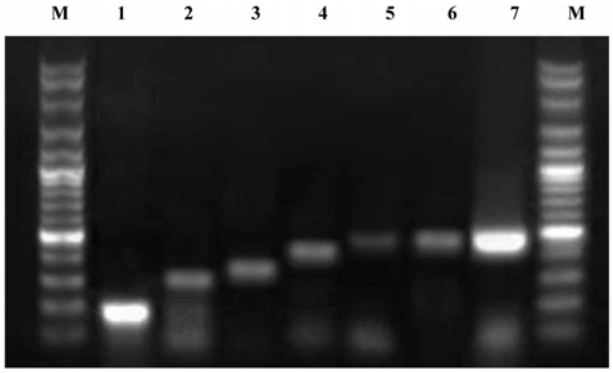

Fig. 1: PCR amplification for the detection of *staphylococcus aureus* SE genes. Lane M: 100 bp marker. Lane 1: sea (180 bp), Lane 2: sej (306 bp), Lane 3: sed (339 bp), Lane 4: seg (432 bp), Lane 5: sei (454 bp), Lane 6: seh (463 bp), and Lane 7: seb (478 bp)

Discussion

Staphylococcus aureus produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism. The SEs are recognized agents of the Staphylococcal food poisoning syndrome (Straub *et al*., 1999). In the present work, nine major types of enterotoxin genes are investigated. Considering the genes encoding enterotoxins, 28 (53.8%) out of 52 isolates of *S. aureus* were positive for at least one enterotoxin gene. The most frequently observed gene was *Sea*, observed in 16 (30.7%) isolates. In spite of the great discrepancy in data concerning the prevalence of enterotoxigenic *S. aureus* isolates found in the literature, which is attributable to the different types of foods and strains involved (Mathieu *et al*., 1991), SEA is the most frequently observed enterotoxin in enterotoxigenic strains of *S. aureus* (Normanno *et al*., 2005). In the current study, it was revealed that genes of *Sea* and *Seb*, the newly described enterotoxin genes of *Seg*, *Seh* and *Sej* seemed to be the predominant enterotoxin genes of *S. aureus* isolated from milk of cows. These data were accordance with the finding of Akineden *et al*. (2001), too. Our study showed that 80.7% of 52 isolates were positive for the presence of genes coding for one or more enterotoxins. In Italy, Morandi *et al*. (2007) found that 67% of the *S. aureus* isolated from milk and dairy products were positive for the presence of toxin genes. In Japan, Omoe *et al*. (2002) observed that 77.4% of the *S. aureus* isolates were positive for the presence of genes that encode one or more enterotoxins, a frequency close to that observed in the present work (80.7%). With the discovery of the new enterotoxins, the percentage of enterotoxigenic or potentially enterotoxigenic *S. aureus* isolates increased. In this study, 28 (53.8%) isolates were positive for at least one *Se* gene, however, that number would decrease to 18 (34.6%) isolates if only the classic enterotoxins (*Sea* to *See*) were considered. Rosec and Gigaud (2002) also observed the increase in the number of enterotoxigenic isolates as a consequence of the discovery of the new SEs. In their study, 30% of the isolates had genes encoding the classic toxins in which frequency was found to be 57% when the new SEs were taken into account. In the present work, six isolates (11.53%) were positive for genes encoding three enterotoxins. Their individual genotypes were *Sea*+*Seb*+*Seh*+, *Sea*+*Seb*+*Sej* and *Seb*+*Sed*+*Seg*+. Nashev *et al*. (2002) have identified genetic profiles comprising multiple genes in *S. aureus*.

In conclusion, the genotypic results of the present study might help to understand the distribution of enterotoxigenic *S. aureus* clones among bovine isolates. This can aid in the investigation and control of *S. aureus* infections in dairy herds.

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