

Presence of enterotoxigenic *Staphylococcus aureus* in cow, camel, sheep, goat, and buffalo bulk tank milk

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RAHIMI, E., F. ALIAN: Presence of enterotoxigenic *Staphylococcus aureus* in cow, camel, sheep, goat, and buffalo bulk tank milk. Vet. arhiv 83, 23-30, 2013.

ABSTRACT

The aim of this study was to determine the ability to produce enterotoxins and enterotoxin types A through E produced among *S. aureus* isolated from bulk tank milk in Fars, Iran by enzyme-linked immunosorbent assay. From September 2010 to July 2011 a total of 200 cow (n = 50), sheep (n = 40), goat (n = 40), camel (n = 30), and buffalo (n = 40) bulk milk samples were collected from 46 randomly selected herds in Fars provinces, Iran. In this study, 22 of 200 raw milk samples (11.0%) were found to be contaminated with *S. aureus*. The highest prevalence of *S. aureus* was found in buffalo milk (17.5%), followed by cow (16.0%), sheep (10.0%), goat (7.5%), and camel (3.4%). The ability to synthesize classical enterotoxins was found in 15 of 22 (68.2%) isolates. Six isolates (27.3%) produced SEA, 4 isolates (18.2%) produced SEC, 3 isolates (13.6%) produced SED, 1 isolate (5.0%) produced SEB, and 1 isolate (5.6%) produced both SEA and SEC (5.0%). No SEE was identified in raw milk samples. This study indicates that the presence of enterotoxigenic *S. aureus* in raw milk may contribute to the sources of staphylococcal food poisoning in Iran.

Key words: raw milk, ruminant milk, staphylococcal enterotoxins, *Staphylococcus aureus*

Introduction

Staphylococcus aureus is one of the most common agents in bacterial food poisoning outbreaks (LONCAREVIC et al., 2005; PELISSER et al., 2009). It is a versatile pathogen of humans and animals and causes a wide variety of diseases, ranging in severity from slight skin infection to more severe diseases such as pneumonia and septicaemia (LOWY, 1998).

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Despite its pathogenicity, *S. aureus* is also harbored in the nares of about 20-30% of healthy people, while about 60% of the population harbors the microorganism intermittently (KLUYTSMAN et al., 1997). *S. aureus* produces different extra-cellular protein toxins and virulence factors, which enhance its pathogenicity due to their enterotoxins (AKINEDEN et al., 2008). *S. aureus* may produce a large variety of enterotoxins (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R and U), and it is common for *S. aureus* to produce one or more of these toxins simultaneously, but 95% of poisoning outbreaks are caused by classical enterotoxins: A, B, C, D and E (PELISSER et al., 2009; LETERTRE et al., 2003).

Staphylococcal enterotoxins (SEs) are a family of major thermostable serological types, which means that the biological activity of toxins remains unchanged even after thermal processing of food and also resistant to gastrointestinal proteases such as pepsin. It can remain active after ingestion (PELISSER et al., 2009; BALABAN and RASOOLY, 2001; ADWAN et al., 2005; CREMONESI et al., 2007). There are many factors affecting enterotoxin production in food such as cell count, salt concentration, pH, temperature and presence of competitive flora (PELISSER et al., 2009; BALABAN and RASOOLY, 2001; CREMONESI et al., 2007; NECIDOVA et al., 2009).

Staphylococcal food poisoning (SFP) is a mild intoxication occurring after the ingestion of food containing from 20 ng to >1 µg of staphylococcal enterotoxin (SE), enough to determine symptoms in human beings (NORMANNO et al., 2007). SFP symptoms appear within a few hours (i.e. 1-6 h) after ingestion of contaminated food, depending on individual susceptibility and toxic dose ingested. Symptoms are characterized by nausea, vomiting, abdominal cramps and diarrhea (JØRGENSEN et al., 2005; NORMANNO et al., 2005). Clinical signs of SFP generally disappear within 24-48 h, while deaths occur rarely, specifically in the very young or elderly. Milk is a good substrate for *S. aureus* growth and among the foods implicated in SFP, milk and dairy products play an important role, since enterotoxigenic strains of *S. aureus* have been frequently isolated in them (PELISSER et al., 2009; BALABAN and RASOOLY, 2001; ADWAN et al., 2005; NORMANNO et al., 2007; JØRGENSEN et al., 2005)

Currently, there is limited information regarding the prevalence and the ability to synthesize classical enterotoxins of *S. aureus* in raw dairy milk in Iran. The aims of this study were to determine the prevalence rate, and enterotoxigenicity, of *S. aureus* in raw cow, sheep, goat, camel, and buffalo milk in Fars, Iran.

Materials and methods

Sample collection. Overall, 46 cow, sheep, goat, camel, and buffalo herds were randomly selected in Fars provinces, Iran. From September 2010 to July 2011 a total of 200 cow (n = 50), sheep (n = 40), goat (n = 40), camel (n = 30), and buffalo (n = 40) bulk milk samples were collected from several individual animals. The samples were

immediately transported to the laboratory in a cooler with ice packs, and were processed within an hour of collection.

Detection of S. aureus. The samples were processed immediately upon arrival using aseptic techniques. To detect *S. aureus*, 1mL of each milk sample was inoculated on Baird -Parker agar (Difco, Detroit, Michigan, USA). After 24 - 48 h of incubation at 37 °C, suspected colonies were sub-cultured on blood agar plate (Difco, Detroit, Michigan, USA) and incubated for 24 h at 37 °C. To identify *S. aureus*, Gram stain, colony morphology, catalase, coagulase, and Voges-Proskaver (VP) tests were conducted on suspected colonies (PELES et al., 2007).

Detection of classical staphylococcal enterotoxins (SEs). To detect SEs, the isolates were cultured overnight aerobically in 10 mL nutrient broth (Merck, Germany) at 37 °C. Bacterial culture supernatants were collected by centrifugation at 4,000 × g for 10 min and used for detection of SEA, SEB, SEC, SED, and SEE using an enzyme linked immunosorbent assay (ELISA) detection kit (RIDASCREEN® SET A, B, C, D, E Art. No: R4101, R-Biopharm AG, Germany). The assay was performed according to the manufacturer's recommendations and as described elsewhere (RAHIMI and GHASEMIAN SAFAI, 2010). The mean lower detection limit of the assay was 0.1 mg/mL. All experiments were performed in duplicate.

Microtiter strips were inserted into the microwell holder (a strip has 8 wells from A -H). 100 µL of the prepared sample was added to wells from A to G of one microtiter strip and 100 µL of the positive control was added to well H, mixed gently by rocking the plate manually, and incubated for 60 min at room temperature. The liquid was completely poured off the wells. The wells were filled with 250 µL of washing buffer and the liquid poured out again (the washing procedure was repeated two times). 100 µL of the diluted enzyme conjugate was added to the wells, gently mixed by rocking the plate manually, and incubated for 60 min at room temperature. The wells were washed 3 times with 250 µL of washing buffer. 50 µL of substrate and 50 µL of chromogen were added to the wells. After incubation for 30 min in the dark at room temperature, 100 µL stop solution reagent was added to each well. The wells were mixed gently by rocking the plate manually and the absorbance was measured at 450 nm using an ELISA reader (Start Fax 2100, UK) with 30 min of the addition of the stop solution. The cut-off value was obtained with adding 0.15 to the mean value of the negative control.

Statistical analysis. Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), chi-square test and fisher's exact two-tailed test analysis was performed and differences were considered significant at values of $P < 0.05$.

Results

Table 1 presents the prevalence and enterotoxigenicity of *S. aureus* isolated from cow, sheep, goat, camel, and buffalo raw milk in Fars provinces, Iran. In this study, 22 of 200 raw milk samples (11.0%) were found to be contaminated with *S. aureus*. The highest prevalence of *S. aureus* was found in buffalo milk (17.5%), followed by cow (16.0%), sheep (10.0%), goat (7.5%), and camel (3.4%) milk. However, no significant differences in the prevalence rates of *S. aureus* were observed between different milk samples ($P < 0.05$). The positive samples were from 3 of 10 (30.0%) commercial dairy herds, 2 of 10 (20%) sheep breeding farms, 2 of 10 (20%) goat breeding farms, 1 of 6 (16.7%) camel breeding farms and 4 of 10 (40%) buffalo breeding farms.

Table 1. Prevalence and enterotoxigenicity of *Staphylococcus aureus* isolated from dairy cow, sheep, goat, camel, and buffalo raw milk samples

Milk samples	No. of samples	<i>S. aureus</i> positive samples	Enterotoxigenic <i>S. aureus</i>	SEs (%)					
				SEA	SEB	SEC	SED	SEE	SEA+SEC
Cow	50	8 (16.0%)	6 (75.0%)	4	1	-	1	-	-
Sheep	40	4 (10.0%)	3 (75.0%)	-	-	2	-	-	1
Goat	40	3 (7.5)	2 (66.7%)	-	-	2	-	-	-
Camel	30	1 (3.4%)	1 (0.0%)	-	-	-	-	-	-
Buffalo	40	7 (17.5%)	4 (57.1%)	2	-	-	2	-	-
Total	200	22 (11.0%)	15 (68.2)	6	1	4	3	0	1

The ability to synthesize classical enterotoxins was found in 15 of 22 (68.2%) isolates by using the ELISA technique. Six isolates (27.3%) produced SEA, 4 isolates (18.2%) produced SEC, 3 isolates (13.6%) produced SED, 1 isolates (5.0%) produced SEB, and 1 isolates (5.6%) produced both SEA and SEC (5.0%). No SEE was identified in raw milk samples. Type A enterotoxin was found in 4 cow milk and 2 buffalo milk samples. Type B enterotoxin was found in 1 cow milk sample. Type C enterotoxin was found in 2 sheep milk and 2 goat milk samples. Type D enterotoxin was found in 1 cow milk, 1 buffalo milk and 1 goat milk samples. Type A and C enterotoxin was found in 1 sheep milk samples.

Discussion

In our survey, the overall prevalence of *S. aureus* in the analyzed samples was 15.2%, with a higher prevalence in buffalo and cow milk. In general, the prevalence rates of *S. aureus* in cow, buffalo, sheep, goat, and camel milk found in this study are comparable with those reported by other investigators (NORMANNO et al., 2007; NORMANNO et al., 2005; MØRK et al., 2010; SHUIEP et al., 2009). However, lower prevalence rates of *S. aureus* in bovine or caprine bulk milk samples (JØRGENSEN et al., 2005; PELES et al., 2007) or

raw milk products (HUONG et al., 2010; RALL et al., 2008) have also been reported. The shedding of bacteria from the infected mammary glands of dairy animals is most likely the primary source of *S. aureus* contamination of milk and dairy products. Contamination may also occur during the cooling, storage, and serving procedures.

Although *S. aureus* is a well known bacterial pathogen in human and animal infections, little information is available at present about the occurrence and the toxinogenic potential of this bacterial species in camel milk and about the role raw camel milk might play in intoxication of the consumer by staphylococcal enterotoxins. In the present study, only 1 of 30 (3.4%) camel bulk milk samples collected from 12 camel breeding farms was positive for *S. aureus*, which did not produce a detectable amount of the classical enterotoxins. Similarity, in a recent study in Sudan, 8.8% of the camel milk samples from 15% of the camels were positive for *S. aureus* (SHUIEP et al., 2009). According to the present results, raw camel milk collected in Iran seems to be, at least at this stage, of minor importance as a vector causing SFP.

In this study, 45.6% of isolated *S. aureus* strains produced enterotoxins. This result is in agreement with those reported by other investigators (NORMANNO et al., 2005; MORANDI et al., 2007). However, some of our findings differed from those reported by other researchers in other countries (NEDER et al., 2011). The distribution of the toxins produced by the enterotoxigenic strains showed that SEA, SED and SEC are frequently produced by isolates collected from raw milk samples. The SEs most commonly involved in cases of SFP are SEA and SED (BALABAN and RASOOLY, 2001). Also, SEC has been recognized as an important cause of SFP associated with the consumption of dairy products (TAMARAPU et al., 2001). Therefore, the results of this study indicate that the presence of enterotoxigenic *S. aureus* in raw milk may contribute to the sources of staphylococcal food poisoning in Iran.

In this study, no SEE was identified in raw milk samples. This is in agreement with previous reports where SEE was not found in milk isolates and in strains isolated from foodstuffs (JØRGENSEN et al., 2005; MORANDI et al., 2007). We could not screen enterotoxins of G, H, I, J, K, L, M, N, O, P, Q, R and U in the present study, however, because commercial ELISA test kits for detecting these new enterotoxins were not available.

Our data show that SEs are in close correlation with the *S. aureus* strain origin. For example, a higher ratio of strains isolated from cow and buffalo milk produced SEA and SED, while the strains isolated from sheep and goat milk produced mainly SEC. These results are in agreement with the data reported from other countries (LONCAREVIC et al., 2005; AKINEDEN et al., 2008; JØRGENSEN et al., 2005; RALL et al., 2008; SCHERRER et al., 2004). However, other studies have shown that SEC is the most frequent enterotoxin

produced by *S. aureus* isolated from cow's milk (JØRGENSEN et al., 2005; NORMANNO et al., 2005; SCHERRER et al., 2004; ARAGON-ALEGRO et al., 2007).

In conclusion, the results of this study highlight the potential risk of consuming raw dairy milk, especially in the absence of strict hygienic and preventative measures to avoid the presence of *S. aureus* isolates and SEs production in milk. It is apparent from the present study that public health and food hygiene practices during milking, transportation, and storage should be implemented in Iran to reduce the risk of *S. aureus* related dairy food poisoning.

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Received: 19 December 2011

Accepted: 27 June 2012

RAHIMI, E., F. ALIAN: Enterotoksični *Staphylococcus aureus* u uzorcima mlijeka krava, deva, ovaca, koza i bivolica uzetim iz cisterni. Vet. arhiv 83, 23-30, 2013.

SAŽETAK

Imunoenzimnim testom određivana je sposobnost proizvodnje enterotoksina i tipova enterotoksina od A do E što ih proizvode izolati bakterije *S. aureus* izdvojeni iz mlijeka sakupljenog u cisternama u Farsu u Iranu. Od rujna 2010. do srpnja 2011. bilo je ukupno prikupljeno 200 uzoraka mlijeka iz cisterni i to kravljeg (n = 50), ovčjeg (n = 40), kozjeg (n = 40), devinog (n = 30) i bivoljeg (n = 40). Mlijeko je potjecalo iz 46 nasumce odabranih stada na području Farsa u Iranu. Od 200 pretraženih uzoraka sirova mlijeka 22 uzorka (11,0%) bila su onečišćena bakterijom *S. aureus*. Najveća prevalencija bakterije *S. aureus* dokazana je u uzorcima bivoljeg mlijeka (17,5%), zatim u uzorcima kravljeg (16,0%), ovčjeg (10,0%), kozjeg (7,5%) te devinog (3,4%) mlijeka. Sposobnost proizvodnje klasičnih enterotoksina dokazana je u 15 od 22 (68,2%) izolata. Šest izolata (27,3%) proizvodilo je stafilokokni enterotoksin A, četiri izolata (18,2%) enterotoksin C, tri (13,6%) enterotoksin D, jedan izolat (5,0%) enterotoksin B, te jedan izolat (5,0%) istodobno enterotoksina A i C. U pretraženim uzorcima sirova mlijeka nije bio dokazan stafilokokni enterotoksin E. Istraživanje je pokazalo da prisutnost enterotoksigenih sojeva bakterije *S. aureus* u sirovu mlijeku može biti uzrok trovanja hranom u Iranu.

Ključne riječi: sirovo mlijeko, preživači, stafilokokni enterotoksini, *Staphylococcus aureus*
