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The detection of classical enterotoxins of Staphylococcus aureus in raw cow milk using the ELISA method

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Abstract: Milk and dairy products are frequently contaminated with exterotoxigenic Staphylococcus aureus, which is often involved in Staphylococcal food poisoning. The aim of the present study is to investigate the presence of classical enterotoxins of S. aureus in raw milk that was produced in Isfahan, Iran. For this purpose, 72 milk samples were collected from the bulk milk tanks of 12 milking farms during 3 different seasons (winter, spring, and summer) and tested for the presence of staphylococcal enterotoxins (SEs) using the enzyme-linked immunosorbent assay (ELISA) method. Out of 72 samples studied, 15 samples (20.8%) were positive for at least 1 SE. Of these, 12 (16.7%) were positive for SEA, 9 (12.5%) for SED, and 6 (8.3%) for SEC. None of the samples was positive for SEB or SEE. Statistical evaluation showed that there were not any significant differences ($P > 0.05$) between the presence of SEs in the milk samples tested in winter, spring, and summer. Further studies should be carried out to investigate the presence of these toxins in different foods and their roles in food poisoning.

Key words: Enterotoxins, milk, Staphylococcus aureus

Staphylococcus aureus is considered the world's third most important cause of food-borne illnesses. The ability of *S. aureus* to grow and produce SEs under a wide range of conditions is evident from the variety of foods implicated in staphylococcal food poisoning. Indeed, milk is a good substrate for *S. aureus* growth and dairy products are known sources of food-borne illness. *S. aureus* contamination can occur through the presence of *S. aureus* in the raw milk itself or during its processing (1-5). Cows are probably the main sources of contamination of raw milk with enterotoxigenic *S. aureus* strains. In particular, cows with subclinical *S. aureus* mastitis can release large quantities of *S. aureus* into their milk (6).

Although pasteurization kills *S. aureus* cells, thermostable SEs generally retain their biological activity (7). Thus, because of the importance of these toxins in the public health and food sectors, an efficient screening method to detect the prevalence of enterotoxic strains in foods is required. Indeed, not all staphylococci produce SE and SE production may be insufficient for the contamination of food products. Traditionally, 7 classic antigenic SE types have been recognized: SEA, SEB, $SEC_{1,}$ $SEC_{2,}$ $SEC_{3,}$

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SED, and SEE. During the 1990s, new SEs (SEG, SEH, SEI, and SEJ) were reported and their genes were described. More recent data resulting from partial or complete genome sequence analyses have led to the description of further "new" SE genes: SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, and SEU. The role of these new SEs in food poisoning has not yet been clarified (8-11).

SEA is the most common enterotoxin recovered from food-poisoning outbreaks in the US (77.8% of all outbreaks) followed by SED (37.5%) and SEB (10%) (12). The intoxication is generally not lethal although the elderly are more susceptible to mortality from food borne-induced gastroenteritis than younger individuals (7). Surveys to detect classical enterotoxins and identify enterotoxin genes in *S. aureus* from milk and milk products have been conducted in many countries including Italy (8), Norway (3), Turkey (9), and Brazil (10). To the best of our knowledge, however, there are no published reports about presence of SEs in various food products in Iran. Therefore, the present study was conducted to investigate the presence of classical enterotoxins of *S. aureus* in raw milk in Isfahan, Iran, using the ELISA method. All reagents were purchased from Merck and of analytical quality. The presence of classical enterotoxins of *S. aureus* in milk samples was detected with ELISA (RIDASCREEN^{Ò} SET A, B, C, D, E Art. No: R4101, R-Biopharm AG, Germany).

A total of 72 samples of raw cow milk were randomly collected from the milk tanks of 6 dairy plants in Isfahan, Iran. Samples were then transferred to the laboratory under refrigeration and stored at temperatures between 0 and 4 °C until being analyzed. Milk samples were centrifuged (10 min/3500 ×*g*/10 °C), and the upper layer of cream was removed by absorption. Once the fat was removed, the milk was diluted to a concentration of 1:20 with distilled water and was filtered using a disposable syringe filter holder. Microtiter strips were inserted in to the microwell holder (a strip has 8 wells from A to H). In each microtiter strip, 100 mL of the prepared samples were added to wells A-G and 100 µL of the positive control was added to well H. The strip was 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 (ethanol 70%) and the liquid poured out again; this washing procedure was repeated 2 times. Next, 100 mL 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 walls were washed 3 times with 250 µL of washing buffer before 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 of 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) within 30 min of the addition of the stop solution. The cut-off value was obtained by adding 0.15 to the mean value of the negative control. In this study, 72 raw cow milk samples were analyzed for SEs with the ELISA. Out of 72 samples studied, 15 samples (20.8%) were positive for at least 1 of the SEs included in the analysis (Table). Of the 15 total positive results, 5 samples (6.9%) were positive for only 1 SE, 8 samples (11.1%) were positive for 2 SEs, and 2 samples (2.8%) were positive for 3 different enterotoxins. The SEA enterotoxin was found to be the most frequently occurring (12 positive samples), followed by SED (9 positive samples) and SEC (6 positive samples).

The rate of contamination varied between the 3 seasons, with 16.7%, 29.2%, and 16.7% of SEs detected in winter, spring, and summer samples,

Table. The distribution of classical Staphylococcus aureus enterotoxins in raw milk (Isfahan, Iran).

Sample tested	Positive samples (%)	Types of enterotoxin (%)					
		A	D	A & C	A & D	C & D	A & C & D
72	15 (20.8)	3	6	2	4	1	2

respectively. Statistical evaluation showed no significant differences ($P > 0.05$) between the SEs of milk samples produced in winter, spring, and summer.

Although Iranian law does not establish limits for the amount of *S. aureus* in milk, it is known that the amount of enterotoxins produced by enterotoxigenic strains achieve levels that are sufficient enough to bring about symptoms of food-borne disease when *S. aureus* concentrations exceed 10^5 CFU/mL (11). The great discrepancy in data found in the literature concerning the prevalence of enterotoxigenic *S. aureus* isolates can be attributed to the different types of foods and biovars involved (12). SEA is the most common enterotoxin recovered from food poisoning outbreaks followed by SED and SEB, and it has been shown that 95% of staphylococcal food poisoning outbreaks are caused by enterotoxins SEA through SEE (7).

Our data shows that 15 (20.8%) out of 72 raw milk samples were positive for at least 1 enterotoxin. The most frequently observed SE was SEA, observed in 12 samples (16.7%), followed by SED (9 samples, 12.5%) and SEC (6 samples, 8.3%). These findings are in agreement with Normano et al. (13), Morandi et al. (8), and Serraino et al. (14), who reported frequent findings of enterotoxins A and D in many cow dairy products. However, some of our findings differed from those reported by other researchers in other countries. Such studies have indicated that SEC was the enterotoxin most frequently produced by the tested cow strains (3,15-17) while SEA was usually typical of human isolates (18). We could not screen these enterotoxins in the present study, however, because commercial ELISA test kits for detecting these new enterotoxins were not available.

Asao et al. (5) reported an outbreak of food-borne disease in Kansai, Japan, where 13,420 people were affected after ingesting skim milk and yogurt (prepared with milk powder) contaminated with 0.38 ng/mL and 3.7 ng/g of SEA, respectively. According to the detection limit (0.2 ng/L) of the ELISA method in our present study, concentration levels of SEs in positive samples were higher than 0.2 ng/L. The results from this investigation may provide valuable information that can be cited by other researchers in future studies. In conclusion, the results of the present study showed the importance of periodically monitoring the level of enterotoxins of *S. aureus* in raw cow milk from Isfahan, Iran. Further epidemiological studies are needed to determine levels of isolated enterotoxigenic *S. aureus* or their toxins in food, and investigations should also be performed to find the relationship between the presence of this pathogen or SEs in food and the ability to cause disease in humans.

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