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Enterotoxigenic *Staphylococcus aureus* in Raw Milk in the North of Palestine

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Abstract: Enterotoxin genes (*sea-see*) in *Staphylococcus aureus* isolates recovered from milk of clinically healthy sheep and cows in the north of Palestine were determined using a polymerase chain reaction (PCR). Thirty-seven (37%) out of 100 *S. aureus* isolates were toxin gene positive. Four strains (10.8%) were sea-positive, 20 (54.1%) were seb-positive, 4 (10.8%) were sec-positive, 6 (16.2%) were *sed*-positive and 3 (8.1%) were *see*-positive. None of these enterotoxigenic isolates carried more than one toxin gene. This study indicates that the presence of enterotoxigenic *S. aureus* in raw milk can contribute to the sources of staphylococcal food poisoning in Palestine.

Key Words: Enterotoxigenic *S. aureus*, staphylococcal enterotoxins, SEs, raw milk, Palestine

Introduction

Staphylococcus aureus is one of the most common agents in bacterial food poisoning outbreaks. It is also a major causative pathogen of clinical or subclinical mastitis of dairy domestic ruminants. Poultry, meat and egg products as well as milk and milk products have been reported as common foods that may cause staphylococcal food poisoning (1). *S. aureus* strains produce a spectrum of protein toxins and virulence factors thought to contribute to the pathogenicity of this organism. The staphylococcal enterotoxins (SEs) have been classified into many different types. These enterotoxins are heat-stable and resistant to the action of digestive enzymes (2). The most common types of these enterotoxins are SEA to SEE. Isolates carrying toxin genes *sea* to *see* are responsible for 95% of staphylococcal food poisoning outbreaks (3). The remaining staphylococcal food-borne disease outbreaks may therefore be associated with other newly identified SEs (4-6). Therefore, the presence of *S. aureus* in food can be considered a potential health risk.

This study was conducted to determine the prevalence of enterotoxin genes A (*sea*), B (*seb*), C (*sec*), D (*sed*) and E (*see*) in *S. aureus* isolates recovered from milk of clinically healthy sheep and cows in the north of Palestine as this has not been investigated previously.

Materials and Methods

A total of 250 raw milk samples (Awassi sheep (n = 120) and Fresian cows (n = 130)) from several major herds in the north of Palestine were used in this study between February and April, 2005. None of these animals were diagnosed with clinical mastitis and had mammary glands without clinical abnormalities and were giving apparently normal milk. Samples were collected into sterilized screw cap sample bottles as described previously (7). One milk sample (10-15 ml) was taken aseptically from each mammary gland after washing with water and cleaning the teats with cotton soaked in 70% ethanol and previous discard of the first 3 streams of milk. The samples were immediately taken in a container containing ice cubes to the laboratory for bacteriological analysis. Each milk sample (30 µl) was surface plated on nutrient agar and was incubated at 37 °C for 24-48 h under aerobic conditions. Ten colonies suspected as *S. aureus* were selected and transferred to individual tubes of nutrient broth. Colonies were subcultured on 5% sheep blood agar (Difco) and mannitol salt agar (Oxoid). Gram stain, culture characteristics and coagulase test using fresh rabbit plasma (tube method) were used for the presumptive identification of all isolates. All coagulase-positive isolates were further tested using the API STAPH-

IDENT, 32 Staph (bioMerieux SA, 69280 Marcy-l'Etoile, France) to confirm identification.

Total DNA was isolated from 1 ml of nutrient broth culture incubated for 18-24 h at 37 °C as described previously with a minor modification (4). Cells were pelleted by centrifugation at 12,000 x g for 10 min, washed twice with 1.0 ml of 0.02 M sodium phosphate buffer ($Na_2HPO_4.2H_2O$) pH 7.4 in 0.9% NaCl and centrifugation at 12000 x g for 10 min. The pellet was resuspended in 200 µl of lysis buffer (1 mM EDTA, 10 mM Tris-chloride, pH 8) with 12 U lysostaphin (Sigma) and incubated for 45-60 min at 37 ºC. Then 4.5 U of proteinase K (MO BIO) was added and the resulting mixture incubated for 45 min at 60 °C, and then for 10 min at 95 °C. The total DNA was spun at 12,000 x g for 15 s and kept at –20 °C for DNA amplification.

The *sea*, *seb*, *sec*, *sed* and *see* gene sequences were detected using the primer pairs described previously (8,9). For PCR amplification, the reaction mixture (30 µl) included 10 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate (PeQLab), 1 X PCR reaction buffer (PeQLab), 1.5 mM MgCl2 (PeQLab), and 1U of Taq DNA polymerase (PeQLab), and finally 1 µl of DNA template was added to each 0.2-ml reaction tube. The amplification was carried out using a thermal cycler (Mastercycler personal, Eppendorf) with the following program: 1 x 4 min precycle at 94 °C, followed by 30 PCR cycles (2 min at 94 °C for denaturation, 2 min at 55 °C for annealing, and 1 min at 72 °C for extension). A final extension incubation at 72 °C for 5 min was used. PCR products were separated by electrophoresis in 2% agarose gel and stained with ethidium bromide. All primers used in this study were synthesized by Integrated DNA Technologies (IDT), Inc., USA.

Results and Discussion

Out of the 100 *S. aureus* isolates (milk sheep origin = 52; milk cows origin = 48) tested for SE-genes, 37 (37%) were positive. None of these enterotoxigenic *S. aureus* isolates carried more than one toxin gene. The majority of these positive toxin gene isolates, 20 (54.1%), were *seb*-positive. The toxin gene profiles of enterotoxigenic *S. aureus* isolates are presented in the Table.

This result was consistent with previous reports from Japan, Poland and Slovakia, where 64% to 85% of the enterotoxigenic *S. aureus* isolates recovered from raw poultry meat or different food samples and food manufacturers harbored the toxin gene *seb* (10-12). On the other hand, our result was in contrast to other studies from Spain, Kenya, Switzerland, Brazil, South Korea, the USA and Slovakia, where most enterotoxigenic *S. aureus* isolates usually carried the toxin gene *sec*, *sea* or *sed* (13-14,15-19).

In the present study, detection of SE-genes by PCR allows the determination of potentially enterotoxigenic *S. aureus* irrespective of whether the strain produces the toxin or not. For this reason, PCR may be considered more sensitive than methods that determine SE-production as immunological methods (12,20). If other newly discovered toxin genes such as *seg, seh,* and *sei*. were detected the prevalence may be higher than previously obtained (6). We know that some of these

Table. Toxin gene profiles of 37 enterotoxigenic *S. aures* isolates recovered from milk samples of clinically healthy sheep and cows in the north of Palestine.

Toxin gene	No. of Enterotoxigenic *S. aureus* strains					
	cow's milk (14 strains)		sheep's milk (23 strains)		Total	
	+ve gene	-ve gene	+ve gene	-ve gene	+ve gene	-ve gene
sea	1 (7.1%)	13 (92.9%)	3 (13.0%)	20 (87%)	4 (10.8%)	33 (89.2%)
seb	6 (42.9%)	8 (57.1%)	14 (60.9%)	9 (39.1%)	20 (54.1%)	17 (45.9%)
sec	2 (14.3%)	12 (85.7%)	2 (8.7%)	21 (91.3%)	4 (10.8%)	33 (89.2%)
sed	4 (28.6%)	10 (71.4%)	2 (8.7%)	21 (91.3%)	6 (16.2%)	31 (83.8%)
see	1 (7.1)	13 (92.9%)	2 (8.7%)	21 (91.3%)	3 (8.1%)	34 (91.9%)

new SEs may be associated with toxic shock syndrome (TSS) and staphylococcal scarlet fever (SSF) (21). The prevalence of enterotoxigenic *S. aureus* in different food samples such as meat, milk, milk products and egg products reported for countries such as Brazil, the USA, Switzerland, South Korea, Poland, Slovakia, China, France and Japan has ranged from 4.7% to 77.4% (4-5,9-14,17-19,22-26). The prevalence of staphylococcal enterotoxin producing strains from milk or other food samples differs among studies. This might be due to differences in the reservoir in the various countries or ecological origin of strains, the sensitivity of detection methods, detected genes and number of samples, and kinds of examined samples included in these studies.

Contamination of dairy products with *S. aureus* may be due to the presence of this pathogen in the basic raw material milk. This is very important, especially in countries producing large amounts of milk products such as cheese. In Palestine, cheese is mostly prepared from unpasteurized cow's and sheep's milk and therefore can contribute to the sources of staphylococcal food poisoning. To our knowledge, this is the first survey to estimate the prevalence of enterotoxigenic *S. aureus* from raw milk used for human consumption in Palestine. It can be concluded that raw milk is contaminated by this pathogen in this area as well as in other countries and might constitute a risk for *S. aureus* enterotoxin food poisoning. Although the number of *S. aureus* isolates tested in this study was not very large, it represents a sample in the north of Palestine, giving a picture of the general situation in this part of the country. Thus, further studies are needed to examine enterotoxigenic *S. aureus* isolates or their toxins in other types of food, and investigations should be performed to find the relationship between this pathogen in food and as a cause of human disease in this country.

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