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The Determination of *S. aureus* and *S.aureus* Enterotoxin - A Producing Strain by Real-Time PCR at Meatball Meals of Two Hospitals in Ankara, Turkey

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This research was planned and carried out in order to determine *Staphylococcus aureus* and Staphylococcal Enterotoxin A presence quickly in meatball meals cooked in two different hospitals with two different cooking systems by using molecular and classical microbiological methods. Total 60 samples of meals with meatballs (30 samples from each hospital) were included in the research. *S.aureus and S.aureus* Enterotoxin A producing bacteria was detected in 6.6% of the samples of hospital A which used new production techniques by using both molecular and classic microbiologic methods. *S.aureus and S.aureus* Enterotoxin A producing bacteria was detected in 9.9% of samples of Hospital B which used traditional methods by using both microbiologic methods. No statistical difference was found out between two microbiologic methods in terms of *S.aureus* identification. Consequently, hygienic rules must be obeyed during all phases, the conditions must be improved and sustainable training of personnel must be ensured in order to prevent food poisoning cases in hospital food service systems. Also, molecular microbiologic methods used for determination of nutrient pathogens are faster and more functional, so usage of these methods is crucial.

Key words: *S.aureus*, Institutional food service, Hospital, Hygiene, PCR.

Today, in accordance to the increasing number of people that are eating outside, the quality of institutional food management systems is changing and also service areas are getting wider[4]. The foods produced without hygiene in those service areas cause food originated diseases.

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Staphylococcus aureus is one of the most frequently associated bacteria related to food originated diseases. *S.aureus* which is a facultative anaerobic gram positive coccus is found in both human and animal ecological indents, especially in nasal cavities of man as well as dairy products, cooked and uncooked meat, etc. The enterotoxins produced by *S.aureus* are resistant to heat and may cause food poisoning characterized by abdominal cramps, nausea, vomiting and sometimes diarrhea[10; 17]. The symptoms usually occur within 30 minutes to 8 hours following

consumption of toxin containing food and subside spontaneously within 24 hours [11].

Staphylococcal entreotoxins play the major role in the pathogenesis of *S.aureus* related gastroenteritis and food poisoning. Currently about 15 staphylococcal enterotoxins (SEs) have been defined. They are mainly classified into 5 major classical types A-E. However, there are newly defined SEs such as SEG, SEI, etc. The most prevalent type causing food poisoning is staphylococcal enterotoxin A (SEA)[9]. The *entA* gene coding SEA is carried by a bacteriophage.

The detection of *S. aureus* and SE in food is often difficult. Food processing may kill the bacteria without destroying the heat resistant SE. Enzyme-linked immunosorbent assays are the currently used classical methods for the direct detection of SE in food samples. These methods are time-consuming, expensive and require a detection limit of SE higher than the level required for staphylococcal food poisoning. A commercially present reversed passive latex agglutination (SET-RPLA) method is more commonly used to detect the presence of SE in bacterial cultures prepared from food samples.

In recent years, molecular microbiological methods have been used more frequently than the classical methods for rapid and reliable identification of microorganisms in various clinical samples[1; 13]. These methods can also be used in the microbiological analysis of food samples[14].

This study was conducted to investigate the presence of *S.aureus* and SEs in the meatball meals of two tertiary care hospitals in Ankara, Turkey by classical microbiological methods and by molecular techniques and also to analyse the efficiency of the two test methods.

MATERIAL AND METHODS

The general properties of inpatient treatment facilities (hospitals)

The study was conducted in two tertiary care hospitals (A and B) in Ankara, Turkey, from January to December 2008. The menus of 2 different hospitals from where the samples were collected are nonselective 3 container sets. While hospital A uses one of the novel manufacturing techniques (C-C) cook – chill, hospital B uses traditional manufacturing techniques -cook fresh- (C-F). Hospital A produces its own meal and has privatized the serving facility while hospital B has privatized all meal production processes and serving.

Collection of Samples

After portioning, food samples containing all food ingredients (200 gr from each) were taken by service staff to sterile sample (2 containers for each) containers before being served to patients. The purpose of this sample collection was to obtain the same quality of food sample that would be served to the patients.

The samples taken to sterile containers placed in carrier cases at 0-4°C, were brought by the researchers to Food Microbiology Laboratory in Hacettepe University Department of Nutrition and Dietetics for the preparation of the homogenate.

Preparation of Homogenate

Two different homogenates to be used in the two different test methods were prepared from the food samples. For use in latex agglutination tests 10 g of sample was taken from the food specimens by using a sensitive scale and placed in 20mL sterile bags and diluted with 0,75% NaCl. The diluted samples were processed by stomacher (IUL, Germany) for 5 minutes and the part passing from the filter was taken into a sterile beaker.

For use in molecular microbiological method 10 g of sample was taken from the food specimens by using a sensitive scale and placed in 20 mL sterile bags including Tris-EDTA solution. The diluted samples were processed by stomacher (IUL, Germany) for 5 minutes and the part passing from filter was taken into a sterile beaker. 1 mL samples were placed into 3 separate screwed tubes and stored at -80 °C for future analysis.

Determination of S.aureus and Staphylococcal Toxins

Classical Microbiological Method

1 mL was taken from the homogenized sample and mixed with 9 mL sterile distilled water and then 10 µL was placed on Baird Parker agar (Oxoid, UK) which was selective for *S.aureus* and also onto sheep blood agar (Oxoid, UK) by diffusion method in aseptic conditions and incubated at 35°C for 48 hours.

Typical colonies with black cambered centers produced in Baird-Parker agar were further evaluated for *S.aureus* identification. Gram

positive cocci which were catalase and coagulase positive were further confirmed by latex agglutination test (Slidex Staph Plus, bioMerieux, France) for the identification of *S.aureus* strains. Colonies determined as *S.aureus* were stored in brain-heart infusion broth including 10 % glycerol at -20°C until used for further tests[15].

For the determination of *SEs*, SET RPLA Toxin Detection Kit (TD 900 Oxoid, UK) based on reversed passive latex agglutination method was used. This kit involved polystyrene latex particles sensitized with purified antiserum taken from rabbits, immunised individually with purified staphylococcal enterotoxins A, B, C and D. The test was carried on in "V" based microplates according to the instructions of the manufacturer. Prepared homogenate was centrifuged at 900 G at 4°C for 30 minutes. In brief, the supernatant was filtered through a 0,2 µm – 0,45 µm low protein binding membrane filter and latex reagents sensitized with antisera to SEA to SED are mixed with supernatant dilutions and incubated overnight and the results were evaluated according to the development of homogenous agglutination in the wells.

Molecular Microbiological Method

DNA Isolation

DNA isolation was conducted by using MagNa Pure LC (Roche Applied Science, Germany) automated nucleic acid isolation device. For DNA isolation from 20-200 µL food samples "DNA III Blood Cells High Performance" protocol was used. 200 µL of bacterial suspensions were placed in suitable sections of the device. The solutions used for isolation were as follows (1-8X); Wash Buffer I 10.8 mL, Wash Buffer II 11.0 mL, Lysis/Binding Buffer 3.6 mL, Proteinase K 1.8 mL, MGP 2.2 mL, Elution Buffer 1.8 mL. 100µL of bacterial DNA was stored at -20°C until used for future analysis.

In this research, MagNa Pure LC isolation technology was preferred as it does not require centrifugation and other manual steps[16].

Amplification of Bacterial DNA by Real-Time PCR

nucA gene was selected as the *S.aureus* specific genomic marker and Real time PCR process was carried on by using FRET ("Florescence Resonance Energy Transfer") probes. The sequences of used primers and FRET probes (Tib Molbiol, Berlin, Germany) for amplification of *nucA* gene were given in Table 1.

Table 1. The sequences of primers and FRET probes used for amplification of *S.aureus nucA* gene

Primer	Gene bank:V01281	%GC	Tm (°C)
nucA	F:AGCCAAGCCTTGACGAACTA A	47.6	58.2
nucA	R:GCGATTGATGGTGATACGGTT	47.6	57.5
FRET probe			
nuc FL:	TGCTTCAGGACCATATTTCTCTACACCTTT	40	62.9
nuc LC:	TAGGATGCTTTGTTTCAGGTGTATCAACCA	40	64.6

PCR process was carried on by using "Light Cycler 2.0" real-time PCR device (Roche Applied Science, Germany). The PCR mixture was prepared by using "Light Cycler Fast Start DNA Master Plus HybProbe" kit protocol. The prepared PCR mixture was placed in the capillary tubes of Light Cycler 2.0 device and then tubes were placed into the device to carry on the real-time PCR process.

S. aureus enterotoxin A production was investigated by PCR amplification of *entA* gene. *entA* gene was detected by real-time PCR carried on by using TaqMan probe. DNA isolation and pre-stages were carried on according to the above protocol. The necessary primer and probes for determination of related gene were obtained from gene bank (code nr L22566). The sequences of used primers and TagMan probes for amplification of *entA* gene are shown in Table 2.

The temperature cycles in the Light Cycler device were programmed so as to fulfill the following conditions: Denaturation: 95°C, 10min; amplification (45 cycles): 95°C, 10 sec denaturation, 55°C, 45 sec bonding, 72°C, 15 sec polymerization; Cooling: 40°C, 30 sec

Table 2. The sequences of primers and TagMan probes used for amplification of *entA* gene (Palomares, *et al*., 2003).

Primer	Gene Bank:L22566	%GC	Tm (°C)
entA	F: TGTGGAGAGACAGGGCACTG	60	58.7
entA	R: CAGTTGTTGGCCATACGGATT	47.6	58.4
TaqMan Probe	TAAGGCCCAAATCTCAGCCATGCATC	50	66.9

Statistical Analysis

The data were analyzed with SPSS software (Statistical Package for the Social Sciences, version 11.5, SSPS Inc, Chicago, IL, USA). Mean data as well as the percentages of data in each category were computed. Cross tabulation and were used to examine the relationships among and between the variables. Statistical significance was set at a p value of <0.05.

RESULTS AND DISCUSSION

Sixty meatball meal samples taken from hospitals were examined by both latex agglutination method and by real-time PCR. *S.aureus and S.aureus* Enterotoxin A producing strains were found in 2 samples by latex agglutination and in 7 samples by real-time PCR in hospital A. In hospital B, *S.aureus and S.aureus* Enterotoxin A producing strains were found in 3 samples by latex agglutination and in 2 samples by real-time PCR (Table 3). There was no statistically significant difference between the two test methods (p>0.05). Bacterial food poisoning represents a substantial public health problem with enterotoxins produced by *S.aureus* being among the most common causes.

Food production systems vary in institutions where public food service is given. Some institutions prefer new, expensive but less labor intensive methods such as cook – chill, cook – freeze, while other institutions continue traditional production which is cook-fresh daily. Both of these methods are safe as far as they are performed under appropriate conditions. Screening of food samples in terms of bacteriological safety is a part of infection control system. In this study, analysis of food samples was performed on meatball meals by both classical and molecular microbiological methods.

For the determination of SEs by classical methods, reversed passive latex agglutination is the most commonly used method. It detects the toxin only if it is expressed in vitro and the result depends on the amount of toxin being produced. According to this method *S.aureus and S.aureus* Enterotoxin A producing strains were found in 6.6% of samples taken from hospital A which was using C-C method and in 9.9% of samples taken from hospital B which was using C-F (n=30) (Table 3).

Table 3. *S.aureus* and *S.aureus* Enterotoxin A producing strains in meatball meal samples taken from two study hospitals by two different test methods

		Real-time PCR (*nucA-entA*)		
Hospital A (NP)[a] (n=30)		Positive	Negative	
Latex agglutination	Positive	2 (%6.6)	-	2
	Negative	5 (%16.5)	23	28
Total		7 (%23.1)	23	30
				(p>0.05)
Hospital B (TP)[b] (n=30)				
Latex agglutination	Positive	3(%9.9)	0	3
	Negative	2(%6.6)	25	27
Total		5 (%16.5)	25	
				30 (p>0.05)

[a] New production method; [b] Traditional production method

Molecular microbiological analysis (*nucA-entA* real-time PCR) revealed that 23.1% and 16.5% of the samples, were positive in hospital A and B, respectively. The higher detection rate of SEA by molecular methods could be attributed to several factors. The production of a sufficient amount of toxin is essential for successful detection of SEs by latex agglutination method. This may be influenced by culture conditions. If enough toxin is not present in the culture filtrate, this assay may lead to false-negative results. Similar results were obtained by Delcenserie *et al.*,[6] who found 51 positive *S.aureus and S.aureus* Enterotoxin A producing strains out of 79 strains by molecular method and 43 positive strains by the classical method. Klotz et al also reported higher detection of SEs by TaqMan PCR than latex agglutination[9]. Morandi *et al*. stated that[12] molecular methods showed a good correlation with classical methods. Although no statistically significant difference was determined between the efficiency of molecular methods and classical methods in terms of detection of SEA positive strains in the food samples, real-time PCR seemed to be much more sensitive than latex agglutination method.

The rate of higher positive results obtained by the molecular method may indicate a possible cross-contamination during institutional food production in hospital A which used cook-chill method. Although novel manufacturing techniques have many advantages, the main disadvantage of these methods is contamination risk since novel manufacturing techniques include more production steps than cook fresh and each step increases the cross-contamination risk if the process is not done under proper conditions. [2; 18], [3; 5]. However, this contamination can be prevented by appropriate cooking and serving methods at the end process. When the results of latex agglutination method was considered, it was observed that SEA positivity rate was lower in hospital A. This might be attributed to the more appropriate cooking method of hospital A which tried to maintain a core temperature above 75°C. It was also stated in previous studies that suitable cooking methods reduced the number of microorganisms significantly in meatballs [2; 19]. Classical microbiological analysis results indicated that all *S.aureus* positive meatball samples obtained from the two hospitals produced SEA. Molecular methods also detected *nucA* and *entA* genes in all *S.aureus* positive samples. However, toxin analysis from food homogenates was not performed in this study. Thus although it was obvious that the detected bacteria in the samples had the ability to produce enterotoxin A, no information were available about the presence of toxins in food samples. Nevertheless, it might be concluded that this toxin producing bacteria defined in meatball meals could produce enterotoxins in suitable conditions and may lead to food poisoning. Since these toxins are resistant to heat, they can not be removed by cooking and may lead to important public health problems. [7; 8]. The results of this study indicated that more *S.aureus* enterotoxin A gene positive samples were detected by real-time PCR. Molecular methods like real-time PCR is well adjusted for screening large numbers of food samples at the same time, allowing rapid, reliable, efficient, and cost-saving detection of microorganisms or their products.

CONCLUSION

Food production systems vary in institutions where public food service is given. Some institutions prefer novel manufacturing techniques but less work power requiring methods such as cook – chill, cook – freeze, while other institutions continue traditional production which is cooking and serving in the same day. Both methods are not risky as far as they are performed under suitable conditions. The foods produced in public food systems should not carry intoxication risk. Some microbiological methods are used to detect the pathogens in foods. The classical method which is culture dependent is considered as gold standard. However, analysis period can last for 3-4 days. Molecular microbiological methods can define the pathogens much faster. Rapid identification of the pathogens results in self-control of the institution, establishment of necessary measures to prevent many food originated diseases. For this, check points should be determined from beginning of the production chain to the end and risk analysis should be made so that the products can be safely consumed.

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