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Prevalence and PCR Sensitivity Comparison of *Toxoplasma gondii*, *Listeria monocytogenes* and *Staphylococcus aureus* in Salads and Appetizers Consumed in Istanbul

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ABSTRACT

This study was conducted to investigate incidence of *Toxoplasma gondii*, *Listeria monocytogenes* and *Staphylococcus aureus* in 100 samples of salad and appetizers (50 salad and 50 appetizer samples) collected from retailers located various districts of Istanbul. While only PCR procedures were used for the analysis of *Toxoplasma gondii*, conventional microbiological methods and PCR procedures were used for analysis of *Listeria monocytogenes* and *Staphylococcus aureus.* Also PCR specify and sensitivity for all the positive samples were calculated. According to the results, 9 (9%) samples had *Listeria monocytogenes*, 36 (36%) samples had *Staphylococcus aureus*, all the samples were negative for *Toxoplasma gondii*. It was noted that PCR sensitivity results were quite specific and accurate for both *Listeria monocytogenes* and *Staphylococcus aureus.* It was concluded that salad and appetizers may seriously threat consumers' health microbiologically if they are processed under poor hygienic conditions.

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İstanbul'da Tüketilen Salata ve Mezelerde *Toxoplasma gondii*, *Listeria monocytogenes* ve *Staphylococcus aureus*'un Prevalans ve PCR Duyarlılık Karşılaştırması

MAKALE BİLGİSİ

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ÖZ

Bu çalışma, İstanbul'un çeşitli semtlerinde bulunan perakendecilerden toplanan 100 adet salata ve meze örneğinde (50 salata ve 50 meze örneği) *Toxoplasma gondii*, *Listeria monocytogenes* ve *Staphylococcus aureus* insidansını araştırmak amacıyla yapılmıştır. *Toxoplasma gondii*'nin analizi için sadece PCR prosedürleri kullanılırken, *Listeria monocytogenes* ve *Staphylococcus aureus* için geleneksel mikrobiyolojik yöntemler ve PCR prosedürleri kullanıldı. Ayrıca tüm pozitif örnekler için PCR spesifitesi ve duyarlılığı hesaplandı. Elde edilen sonuçlara göre 9 (%9) örnekte *Listeria monocytogenes*, 36 (%36) örnekte *Staphylococcus aureus* tespit edildi, tüm örneklerde *Toxoplasma gondii* negatif olduğu gözlendi. PCR duyarlılığı sonuçlarının, *Listeria monocytogenes* ve *Staphylococcus aureus* için oldukça spesifik ve doğru olduğu tespit edildi. Salata ve mezelerin, zayıf hijyen koşulları altında işlem görmesinin tüketicinin sağlığını mikrobiyolojik olarak tehdit edebileceği sonucuna varıldı.

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Introduction

Foodborne pathogens and parasites are evaluated as important risk factors for public health in both developed and developing countries because of their worldwide spread properties (Dümen and Sezgin, 2009). Almost all foodborne pathogens and parasites may contaminate to the animal and herbal based foods in different stages of the production processes by primary and/or secondary contamination sources (Borch and Arinder, 2002). The salads on retail sale in Turkey include fresh or boiled vegetables with or without cooked chicken meat or canned tuna fish and mayonnaise. The vegetables can be obtained from the fresh products through selection, washing, peeling, cutting, rinsing and packaging, but these processes may not be enough hurdles for the contamination and growth of pathogens and spoilage microorganisms during storage under refrigeration (Pamuk et al., 2013).

Toxoplasma gondii (*T. gondii*) is one of the most prevalent zoonotic parasites in the world (Berger-Schoch et al., 2011). Toxoplasmosis is indicated as the parasitary zoonosis which has the highest incidence in humans. Toxoplasmosis infections in human generally take shape by the intake of the sporulated oocystes via contaminated soil, water and vegetables and feces particles of the cats, consuming of raw/inadequate cooked meats and transplasental passes from mother to fetus. Also, one of the most common contamination sources of acute toxoplasmosis is formed by intake of contaminated fresh water (Bowie et al., 1997).

Staphylococcus aureus (*S. aureus*), is the third main bacteria which cause food toxications in the world (Acco et al., 2003). Toxications are generally formed by intake of the enterotoxins via alimentary way. Although *S. intermedius* and *S. hyicus* are included into the enterotoxigenic *Staphylococcus* species, *S. aureus* is the predominant agent for the food intoxications (Vazquez-Boland et al., 2001). The toxins of *S. aureus* are thermostable molecules. The agent may be inactivate by different heat treatment applications, however, in the case of toxin production of *S. aureus* under appropriate conditions before heat treatment applications toxins cannot be eliminated. Thus *S. aureus* intoxications may threat public health seriously (Bryan 1980).

Listeria monocytogenes (*L. monocytogenes*), is an important food borne pathogen that cause gastroenteritis, septicemia, central nervous system infections, materno-fetal infections and abortions in humans. *L. monocytogenes* is a Gram positive facultative anaerobic microorganism and can be isolated from almost all foods. The agent is identified as the main factor of epidemic and sporadic listeriosis since 1980s by the medical literatures. However listeriosis outbreaks are relatively less common than the other foodborne pathogen based outbreaks. Due to high rate of mortality (up to 40%) *L. monocytogenes* is one of the most dangerous pathogens that threaten public health (Beckers et al., 1987; Fernandez et al., 1986).

The aim of this study was to determine the existence of *T. gondii*, *S. aureus* and *L. monocytogenes* in ready to eat salad and appetizer samples which are sold in the restaurants located in different districts of Istanbul by the conventional microbiological analysis methods and PCR procedures.

Material and Methods

Sampling

100 salad and appetizer samples (50 salad samples and 50 appetizer samples) were collected from the restaurants that were located in various districts of Istanbul. Table 1 show the details of sampling procedure.

Microbiological Analysis

S. aureus: S. *aureus* was determined by surface plating on BPA (Baird Parker Agar) (Oxoid, CM 0961) supplemented with egg yolk-tellurite emulsion (Oxoid, SR0054). Spread plates were incubated at 35°C for 46-48 h. Colonies with typical S. *aureus* morphology were examined microscopically following Gram staining and tested for catalase and coagulase activity (US FDA 2001).

L. monocytogenes: It has been studied according to FDA. Suspected isolates have been defined according to; mannitol, malt sugar, xylose fermentation, Gram staining, catalase, movement, dextroglycoside, rhamnose, nitrate reduction, esculin hydrolysation properties (US FDA 2001).

PCR

DNA of all isolates were extracted according to the protocol of the manufacturer (Macherey-Nagel, Nucleospin® Tissue). All the extracts were stored at -20°C until they are used as target DNA for the PCR procedure. *T. gondii* specific *B1*, *L. monocytogenes* specific *act*A and *S. aureus* specific *nuc* and *coa* genes were reproduced by using specific designed primers (Table 2 shows the specific primer sets that were used in this study). Each PCR mixture was 50 µL and consisted of 2 µL of each primers (forward & reverse), 5 µL 10X buffer (Kapabiosystems, 1.5 mM Mg for the final concentration), 4 µL 25 mM dNTP mixture (TaKaRA), 0.5 µL Taq polymerase (5 u uL^{-1} - Kapa), 34.5 µL distilled water and 2 µL target DNA and the mixtures were put to the thermal cycler. After 1st denaturation at 94°C 2 min, total of 35 cycle (1 min at 94°C, 1 min at 50°C, 1 min at 72°C) and final synthesis step (10 min at 72°C was applied). PCR products were stored at 4°C up to electrophoretic separation. Electrophoresis procedure was applied to 10 µL of PCR products with 2 µL 6X loading buffer at 1.5% agarose with ethidium bromide and existence of specific bands were explored via UV observed bands at 94 bp for *T. gondii*, 827 bp for *L. monocytogenes* and 416 bp for *S. aureus* were evaluated as positive.

Table 1 Details of the collected samples

City	District	Sample name	NS	Sales point type	Explanation
İstanbul	Europe	Salad	25	Restaurant/kebab shop/supermarket	Ready to eat and unpackaged
İstanbul	Anatolia	Appetizer	25	Restaurant/kebab shop/supermarket	Ready to eat and unpackaged
İstanbul	Europe	Salad	25	Restaurant/kebab shop/supermarket	Ready to eat and unpackaged
İstanbul	Anatolia	Appetizer	25	Restaurant/kebab shop/supermarket	Ready to eat and unpackaged

NS: Number of samples

Table 2 Specific primer sets that were used in the study

Primer No	Sequence (5'-3')	Target gene/Amp (bp)	Target microorganism
1	GCTGATTTAAGAGATAGAGGAACA	*actA* 827	*L. monocytogenes*
2	TTTATGTGGTTATTTGCTGTC	*actA* 827	*L. monocytogenes*
3	GGCAATTGTTTCAATATTAC	*nuc*/416	*S. aureus*
4	ATAGAGATGCTGGTACAGG	*coa/500 - 650*	*S. aureus*
5	GCTTCCGATTGTTCGATGC	*coa/500 - 650*	*S. aureus*
6	TTTTATTTGCATTTTCTACC	*nuc*/416	*S. aureus*
7	CACAGAAGGGACAGAAGT	*B1/94*	*T.gondii*
8	TCGCCTTCATCTACAGTC	*B1/94*	*T.gondii*

PCR Specification and Sensitivity

The sensitivity of PCR methods is defined as the ratio of PCR products obtained to the isolated cultures by reference methods (Dohoo et al., 2003). In this study, the relative sensitivity (SE) and relative specific (SP) grades of the applied PCR methods were calculated using the following formulas:

$$SE=\frac{\text{PA value of PCR and reference culture methods}}{N+}\times 100$$

PA : Positive Agreement

N+ : Number of positive samples obtained with reference isolation/identification methods

SP : NA value of PCR and reference culture methods/N × 100

NA : Negative Agreement

N- : Number of negative samples obtained with reference isolation/identification methods (Hyungkun et al., 2005).

For determination of PCR sensitivity, reference *L. monocytogenes* and *S. aureus* strains were serially diluted with 0.1% peptone water (Oxoid CM 009) up to 10^{-9} concentration level (1-10 kob mL-1) so that 5 replication. Grown strains were evaluated as 10^{-9} dilutions of that *L. monocytogenes* and *S. aureus* and the strains were passage to Nutrient Agar (NA) (Oxoid, CM003), including 7 grams L^{-1} yeast extract (Oxoid CM 019). Additionally, a non - *Listeria* and non - *Staphylococcus* mixture consisted of 5 different non - *Listeria* and non - *Staphylococcus* strains were treated with 0.1% peptone water up to 10^{-4} dilution concentration. For each *L. monocytogenes* and *S. aureus* dilution, 1 mL of non - *Listeria* and non - *Staphylococcus* mixture were added to the tubes that included *L. monocytogenes* and *S. aureus* and the bacterial mixtures were incubated at 37ºC for 24 h. After the incubation period, each mixture was passed to BLEB (Buffered Listeria Enrichment Broth Base) (Oxoid, CM0862) (for *L. monocyotgenes)* and NA (for *S. aureus*) of 10 mL. Then, a last incubation at 37ºC for 24 h was applied to mixtures and 1 mL of final mixtures for each sample was stored for PCR procedures. Ten PCR replications for each dilution were applied, and the optimal dilution rate was calculated according to the procedures explained.

Results and Discussion

In this study, 100 salad and appetizer samples sold in different types of sales points located in various districts of Istanbul were analyzed for *T. gondii*, *L. monocytogenes* and *S. aureus. L. monocytogenes* and *S. aureus* were Before isolated and identified by using conventional microbiological analysis and verified by PCR procedures while *T. gondii* was directly analyzed by PCR (Figure 1). Table 3, shows results. *S. aureus* is one of the most common foodborne pathogens that is isolated from different kinds of foods. The agent uses various contamination sources in the food industry. Besides, the agent's resistance capacity to the antibiotics is being increased day by day. Thus, *S. aureus* is still one of the most important foodborne pathogens that threat public health (Livermore 2000; Pesavento et al., 2007). *S. aureus* widespread feature in foods is generally originated from direct and/or indirect contact to foods of contaminated staff. Contaminated hands, noses, mouths of the staff, contaminated food contact surfaces and equipments are identified as the most used secondary contamination ways by *S. aureus*. Furthermore, the agent may also contaminate to the foods from abscesses, acnes and infected wounds of people and/or animals under various conditions. Nose is the most *S. aureus* including organ in adults. Thus, human has a very important role in the human-food and food-human contamination chain. In the food plants which have poor hygienic conditions *S. aureus* infection risk is increased generally. In spite of the development of food sciences and prevention applications in food industry the incidence of the agent in the food is being increased properly anyhow. This situation makes it necessary to produce qualified and microbiologically safe food stuff (Hacıbektaşoğlu et al., 1993).

The studies about existence of *S. aureus* in salads and appetizers are quite limited in our country. Arıcı et al. (2003) specified that they isolated *S. aureus* from 15 of 22 ready to eat salad samples with the up to the concentration of $2.8x10^3$ cfu g^{-1}. Aycicek et al. (2004) analyzed 70 salad samples and they indicated that they have isolated coagulase (+) *S. aureus* from 9 samples up to the concentration of 10^4 cfu g^{-1}. The results and *S. aureus* concentrations that we determined in the study are higher than the results of the aforementioned researchers. According to the results of the study, 36 salad and appetizer samples were positive for *S. aureus.* The highest concentration of *S. aureus* was $1.2x10^7$ cfu g^{-1}. While the lowest concentration was 2.1×10^2 cfu g^{-1}. Among the positive determined samples. Due to the results of the study it is thought that the positive samples may be contaminated from contaminated hands, contact surfaces and equipments as explained above. Salads and appetizers are vegetable based food stuffs, so they may be contaminated primary contamination sources as soil, water, air, etc. by their nature. The agent might use one or more of the said ways to contaminate to the salads and appetizers.

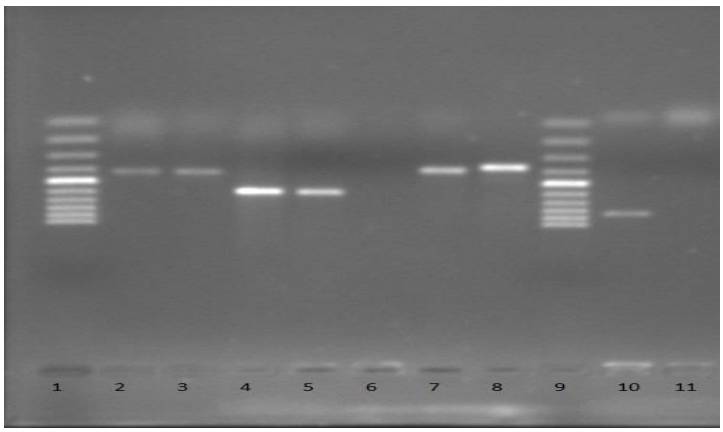

Figure 1 Agarose gel electrophoresis of the amplified products form positive samples. 1: 100bp sm, 2-3: *S. aureus nuc* gene (thermonuclease activity) 416 bp, 4-5: *S. aureus coa* gene (coagulase activity) 500 -650 bp, 6: Negative control (-), 7-8: S. aureus spa (protein A) gene 100 -450 bp, 9: 100 bp sm 10: *L. monocytogenes actA* gene 827 bp, 11: *L. monocytogenes* Negative control.

Table 3 Details of the results that were determined in the study

Analyzed parameter	SN	C/D	SPT	NPS	Explanation
T. gondii	Salad	IE	RKSS	0 (0%)	Not isolated
T. gondii	Salad	IA	RKSS	0 (0%)	Not isolated
T. gondii	Appetizer	IE	RKSS	0 (0%)	Not isolated
T. gondii	Appetizer	IA	RKSS	0 (0%)	Not isolated
L. monocytogenes	Salad	IE	RKSS	3 (6%)	1 sample from supermarket and 2 samples from kebab houses
L. monocytogenes	Salad	IA	RKSS	3 (6%)	3 samples from kebab houses
L. monocytogenes	Appetizer	IE	RKSS	2 (4%)	1 sample from restaurant and 1 sample from supermarket
L. monocytogenes	Appetizer	IA	RKSS	1 (2%)	From supermarket
S. aureus	Salad	IE	RKSS	12 (24%)	4 samples from supermarkets, 6 samples from kebab houses and 2 samples from restaurants
S. aureus	Salad	IA	RKSS	16 (32%)	10 samples from kebab houses and 6 samples from supermarkets
S. aureus	Appetizer	IE	RKSS	2 (4%)	2 samples from kebab houses
S. aureus	Appetizer	IA	RKSS	6 (12%)	3 samples from kebab houses, 2 samples from supermarkets and 1 sample from restaurant

SN: Sample Name, C/D: City - District , IE: İstanbul - Europan, IA: İstanbul – Anatolian, NPS: Number of positive samples, SPT: Sales point type, RKSS: Restaurant/kebab shop/supermarket

Medical literatures indicate that *S. aureus* is easily suppressed in mixed cultures (Acco et al., 2003; Erol 2007; Gündoğan et al., 2005). Besides, it is also stated that lactic acid bacteria which are found in fermented foods also suppress *S. aureus* by the biological products as hydrogen peroxide, bacteriocins and different kinds of antimicrobial components (Acco et al., 2003). The results of our study is parallel to the before mentioned comments. Due to the results we got, 47.2% of the *S. aureus* positive salad and appetizer samples were negative for coliforms, *E. coli*, *L. monocytogenes* and *Salmonella* spp. (data not shown). The salad and appetizer samples might be possibly contaminated by *S. aureus* in the plants via various contamination sources. Another alternative is the purchasing of already contaminated raw materials for preparing salads and appetizers of the food plants. In this case, *S. aureus* may easily reproduce and reach the toxin producing concentrations. It is remarked that *S. aureus* can produce toxins when they reach 10^5-10^6 cfu g^{-1} concentrations (Erol 2007). This situation increases the risk factors of the agents especially for neonate, pediatric, geriatric and immune system suppressed cases (HIV transporters, metabolic disease patients, alcohol/drug/users, patients suffer from auto immune disorders, etc.) who are identified as primary risk group for *S. aureus.*

L. monocytogenes is an important and fatal foodborne pathogen cause septicemia, central nervous system infections, materno-fatal disorders and abortions in humans. The agent has quite high rate of mortality and this situation cause the agent to be identified as the one of the most risky foodborne pathogens all around the world (Mead et al., 1999). Because of ubiquity feature, *L. monocytogenes* can easily survive and reproduce in meat, meat products, milk, milk products, vegetables and vegetable based products (Gugnani 1999). In the United States it was reported 6 big listeriosis outbreaks in between 1979 -1999. The researches about the outbreaks exposed that the main contamination sources were green-leaved vegetables/salads, carrots, potatoes, pasteurized milk, pork and different kinds of cheese. Infected cases suffered from meningitis and encephalitis combined with septicemia and 20% of infected cases died (Vazquez-Boland et al., 2001).

L. monocytogenes infects 2500 person every year in United States and approximately 500 of these infected cases die (Mead et al., 1999). The outbreak salad/vegetable based in Canada in 1980 with 41 case, the outbreak salad and liver pate based in England in 1987-1989 with 355 cases, and the outbreak sandwich and salad based in the united States with 101 cases are the biggest reported outbreaks in the world. However, the sum of annually reported individual cases are not lower than the cases that were infected in outbreaks. In the United States it was reported 696 listeriosis cases in 2003. In 2004 and 2005, these numbers were increased to 753 and 842 cases respectively (CDC 2003; CDC 2004).

According to the results of the study 9 samples were positive for *L. monocytogenes.* The highest concentration was $4.8x10^2$ cfu g^{-1} while the lowset concentration was 8 cfu g^{-1} in positive samples. Because *L. monocytogenes* is a zero-tolerant foodborne pathogen (Mead et al., 1999) all the positive samples were evaluated as "very risky" for consumers' health. In the study *L. monocytogenes* positive samples consisted of unpackaged products with rate of 77.7% (7 samples out of 9). Due to the results obtained it is predicted secondary contamination sources as staff, contact surface and equipments may have important roles for cross contamination of *L. monocytogenes.* According to Ak et al. (1994), *L. monocytogenes* has the ability of cross-contamination to various kinds of contact surfaces in 3 minutes after its Before contamination. However, the positive samples might be already contaminated when they were purchased by the plants. In either case, it can be said that good hygiene and manufacturing procedures and applying food security systems to all kinds of foods at all steps of production period from raw material to consumption, is the key factor for maximizing the microbiological quality of foods and public health.

PCR sensitivity was determined as 19 cells for *L. monocytogenes*. The main factors that make the isolation and identification of *L. monocytogenes* difficult; the accuracy and sensitivity of dilution, and the long duration of isolation (Borch and Arinder, 2002). Because of the reasons explained, it was thought that correct PCR procedures may be a good alternative to microbiological methods for an exact identification for *L. monocytogenes*. The PCR procedures that we used gave quite specific results for *S. aureus*, too. Sensitivity of PCR procedures was determined as 32 cells for the aforementioned agent. By specification of target primers, *S. aureus* positive evaluated samples by microbiological methods exactly matched with the PCR results while the other *Staphylococcus* strains positive results did not (data not shown). Moreover, the results can be obtained average 5 days earlier by PCR procedures when compared with conventional microbiological methods. Because *T. gondii* were not isolated from any of the samples collected, PCR specification procedures were not applied to the abovementioned microorganism. According to our study, PCR results were quite specific and sensitive for *L. monocytogenes* and *S. aureus*. For the identification of mentioned two strains, PCR procedures may be a good alternative to microbiological isolation and identification methods.

The studies about the existence of *L. monocytogenes* in foods except meat, milk and their products are very limited. Results of the study showed that 9% of the analyzed salad and appetizer samples (9 of 100 samples) were positive for *L. monocyotgenes.* According to the results of the study existence risks of *L. monocytogenes* in salads and appetizers which are widely consumed in our country must be taken into consideration. It is estimated that the studies about existence of *L. monocytogenes* in different kinds of foods except meat, milk and their products would be useful for protecting public health.

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