Presence of *Salmonella* in retail grade a eggs determined by the International Organization for Standardization 6579 method and a LightCycler polymerase chain reaction system

Seran TEMELLİ[1], Serpil KAHYA[2], Zafer ATA[1,3], Kamil Tayfun CARLI[2] , Aysegul EYIGOR[1]

Uludag University Faculty of Veterinary Medicine,[1] Department of Food Hygiene and Technology, [2] Department of Microbiology, Bursa,; [3]Military Veterinary School and Educational Central Commandership, Gemlik, Bursa, Turkey.

Summary: This study aims to determine the presence of *Salmonella* in naturally contaminated grade A eggs by the standard culture method International Organization for Standardization Method 6579 (ISO) and a specific real-time PCR system (LightCycler PCR-LCPCR) to complement ISO. A total of 1635 eggs pooled into 101 samples were randomly collected within one year period from 20 different retail markets in Bursa, Turkey, carrying eggs of 16 large egg producers/suppliers of 5 cities with intensive layer production. Preparation of the egg and shell for analyses, *Salmonella* isolations and identifications, and detections were performed according to ISO 6887-4:2003, ISO 6579 and LCPCR, respectively. Overall *Salmonella* detection rate by ISO and LCPCR were 15.8 % (16/101) and 46.5 % (47/101), respectively. Out of 101 inner parts, *Salmonella* was detected in 11 (10.9 %) samples by ISO, and in 31 (30.7 %) samples by LCPCR. Six of 101 shell samples (5.9 %) were found to harbor *Salmonella* by ISO, while 18 (17.8 %) shells were positive by LCPCR. All isolates were determined as *Salmonella enterica* subsp. *enterica* serovar Enteritidis. These findings indicate considerably high *Salmonella* contamination in retail grade A eggs. This should be under routine monitoring by rapid methods such as PCR, complemented by standard culture to evaluate and assess the significance of risk for public health.

Key words: ISO 6579, real-time polymerase chain reaction, retail egg, *Salmonella*.

Perakende A sınıfı yumurtalarda *Salmonella* varlığının ISO 6579 metodu ve LightCycler polimeraz zincir reaksiyonu ile belirlenmesi

Özet: Bu çalışmada doğal kontamine A sınıfı yumurtalarda *Salmonella* varlığının ISO 6579 standart kültür metodu ve bu metodun spesifik gerçek zamanlı bir PCR sistemi (LightCycler PCR-LCPCR) ile desteklenmesini belirlemek amaçlanmıştır. Bursa'da 20 farklı perakende marketten 1 yıl süresince, yoğun yumurta üretimi olan 3 bölge (Ege, Marmara, Orta Anadolu)'nin büyük üretici/tedarikçisi olan 16 farklı firmaya ait 101 birleştirilmiş, toplam 1635 adet yumurta rastgele olarak örneklendi. Yumurtaların analize hazırlanması, *Salmonella* izolasyonu ile identifikasyonu ve deteksiyonu sırasıyla ISO 6887-4:2003, ISO 6579'a göre ve LCPCR ile yapıldı. Yumurtalarda genel *Salmonella* deteksiyon oranları ISO ve LCPCR ile sırası ile %15.8 (16/101) ve % 46.5 (47/101) olarak belirlendi. Yüzbir iç örneğinin ISO ile 11'inde (% 10.9), LCPCR ile 31'inde (% 30.7) *Salmonella* saptandı. Kabuk örneklerinden 6'sında (% 5.9) ISO ile *Salmonella* bulunurken, 18'i (17.8 %) LCPCR ile pozitif olarak tespit edildi. Tüm izolatların *Salmonella enterica* subsp. *enterica* serovar Enteritidis olduğu belirlendi. Bu bulgular perakende A sınıfı yumurtalarda *Salmonella* kontaminasyonunun oldukça yüksek olduğunu ve bu durumun halk sağlığı açısından önemini belirlemek ve değerlendirebilmek için standart kültür metodu ile desteklenen PCR gibi hızlı bir metotla rutin olarak izlenmesi gerektiğini göstermektedir.

Anahtar sözcükler: ISO 6579, gerçek zamanlı polimeraz zincir reaksiyonu, perakende yumurta, *Salmonella*.

Introduction

Salmonella is known as the most important cause of foodborne bacterial enteritis in many countries. Besides the high morbidity and mortality rates in food-borne salmonellosis, food recalls and withdrawals are known to cause significant economical losses in the food industry (22). European baseline studies of 2005-2006 reports *Salmonella* spp. infection rate in European Union as 30.7% in layers (6). The most important sources in *Salmonella* infections and outbreaks are contaminated poultry meat, egg and egg products (25). Many studies performed in different countries and in our country indicate that egg and egg products were contaminated with *Salmonella* in various rates (1, 19), and lead to *Salmonella*-related outbreaks (23).

Rapid detection of *Salmonella* in eggs and its products are of great significance in preventing food-borne salmonellosis. However, Gold Standard culture methods (e.g. International Organization for Standardization Method 6579 [ISO], United States Food and Drug

Administration's Bacteriological Analytical Manual Chapter 5: *Salmonella*), which require up to 5 days (including biochemical and serological confirmations) (11, 15), do not suffice in routine and rapid monitoring of these samples. In recent years, novel real-time PCR assays were developed for the rapid detection of *Salmonella* particularly from eggs (5). LightCycler PCR (LCPCR) is a specific real-time PCR system enabling rapid and reliable detection of the specific PCR product with probe-based technology and high sensitivity (3). Still, complementation of PCR by standard culture is required for the elimination of possible false negative and/or variable PCR results related to inhibitory substances within the process (29), and for avoiding false positive results due to amplification of target DNA from dead/non-culturable/injured *Salmonella* cells in the sample (20).

We conducted this study to determine the presence of *Salmonella* and its possible risk on public health in retail grade A eggs in Bursa, which were produced mainly in 5 cities (Balıkesir, Bursa, Manisa, Sakarya, Ankara) in Turkey by the 'internationally-recognized' standard culture method ISO 6579 (ISO) and a rapid specific real-time PCR system (LightCycler PCR-LCPCR) to complement ISO in detecting *Salmonella* from naturally contaminated eggs.

Materials and Methods

Salmonella strains: SE 64K (M.Y. Popoff, Institut Pasteur, 28 rue du Dr Roux, 75015 Paris Cedex 15, France), and *Salmonella enterica* subsp. *enterica* serovar Typhimurium NCTC 12416 (Refik Saydam National Public Health Agency, Ankara, Turkey) were used as positive controls in ISO and LCPCR tests.

Sampling strategy in collection and analysis of egg samples: A total of 1635 eggs were collected and were pooled into 101 samples (1 sample ≥ 12 egg/viol) were randomly collected from 20 different retail markets in Bursa, which carry eggs of 16 large egg producers/ suppliers of 5 cities with intensive layer production, within one-year period (Table 1). Analysis of all samples was initiated immediately after transfer to the laboratory.

Culture: Two groups of randomly selected-6 eggs from each sample group were used for the culture of *Salmonella* from inner part and shell of the egg. Eggs used for culture from inner part were handled by using sterile gloves and prepared by the method described in ISO 6887-4:2003, Chapter 9.6.1.2 (16) for analysis. Isolation and identifications were performed as indicated in ISO 6579 (15). Serotyping was performed on the basis of reaction with O- and H-group antigen, according to the White- Kauffmann-Le Minor Scheme (13) and in Guibourdenche et al. (14) by using commercial antisera (Becton Dickinson). Eggs to be used for culture from shell were handled by using sterile gloves and prepared

by the 'Soaking method' described in ISO 6887-4:2003, Chapter 9.6.2.4. (16). Isolation and identifications were performed as indicated above in the culture from inner part.

DNA isolation and LCPCR: DNA from 1 ml aliquot taken from the primary enrichment step of ISO 6579 (15) for each shell and inner part egg sample was isolated by using Foodproof Sample Preparation Kit I (Biotecon, 1.20473.0001, Germany). Isolated DNA was used as template in LCPCR (LightCycler 2.0 Instrument, Roche, 03531414201, Germany), which was performed by using a Foodproof *Salmonella* Detection Kit (Biotecon, 1.20453.0001, Germany) after concentration and purity determination by NanoDrop Spectrophotometer (Thermo, ND1000, USA). The total PCR reaction volume was 20 μl comprised of 5 μl of template DNA added into 15 μl PCR mix (13 μl of Foodproof *Salmonella* Master Mix [ready-to-use primer and hybridization probe mix], 1 μl of Foodproof *Salmonella* Enzyme Solution [FastStart Taq DNA Polymerase and Uracil-DNA Glycosylase, heat labile, for prevention of carry-over contamination], 1 μl of Foodproof *Salmonella* Internal Control. The Foodproof *Salmonella* Control template DNA, and DNA from one of the selected *Salmonella* strains indicated above were used as positive controls; and PCR-grade water was used as a negative control in PCR. The amplification protocol and data analysis was performed as indicated in the kit insert and by the LightCycler software version 4.1, respectively.

Statistical analysis: Relative accuracy, sensitivity, and specificity were calculated according to the protocol described in ISO 16140 (17). Relative accuracy, sensitivity, and specificity calculations were complemented with Cohen's kappa test to evaluate the correspondence between results obtained by methods.

Results

In this study, the overall *Salmonella* detection rate by ISO and LCPCR are 15.8 % (16 out of 101 samples) and 46.5 % (47 out of 101 samples), respectively. Out of 101 inner parts, *Salmonella* was recovered in 11 (10.9 %) samples by ISO, compared to 31 (30.7 %) inner part positive samples by LCPCR. Six of the 101 shell samples (5.9%) were found to harbor *Salmonella* by ISO, while 18 (17.8 %) shells were positive by LCPCR (Table 1).

A total of 10 samples comprised of 6 inner (sample no 78, 80, 83, 85, 96, 97) and 4 shell (sample no 12, 82, 88, 93) parts were found *Salmonella* positive both by ISO and by LCPCR. There were 6 samples (sample no 73, 86, 89, 92, 94, 95) detected only by ISO, and 37 samples detected only by LCPCR. One sample (sample no 92) and 2 samples (sample no 4 and 97) were positive for *Salmonella* by ISO, and by LCPCR both in their inner and shell parts (Table 1).

Table 1. Summary of egg sampling strategy and results of culture (ISO) and LCPCR.
Tablo 1.Yumurta örnekleme stratejisi özeti ile kültür (ISO) ve LCPCR sonuçları.

Sampling period	No of samples	City	Supplier	Retailer	No: eggs sampled	Inner positive sample ID		Shell positive sample ID	
						ISO	LCPCR	ISO	LCPCR
Jun11	1	Bursa	A	a	15	-	-	-	-
	2	Bursa	A	a	15	-	-	-	-
	3	Bursa	A	a	15	-	-	-	-
	4	Sakarya	B	b	10	-	4	-	4
	5	Sakarya	B	b	10	-	-	-	-
	6	Manisa	C	c	10	-	-	-	-
	7	Manisa	C	c	12	-	-	-	-
	8	Manisa	C	c	12	-	-	-	-
Jul11	1	Manisa	C	c	12	-	-	-	-
	2	Manisa	C	c	12	-	-	-	-
	3	Bursa	D	d	15	-	11	-	-
	4	Ankara	E	d	15	-	-	12	12
	5	Manisa	C	d	12	-	-	-	-
	6	Bursa	F	e	12	-	-	-	-
	7	Bursa	F	e	12	-	-	-	-
Aug11	1	Bursa	F	e	15	-	-	-	-
	2	Manisa	C	f	10	-	-	-	-
	3	Manisa	C	f	10	-	18	-	-
	4	Bursa	G	g	10	-	19	-	-
	5	Bursa	H	g	10	-	-	-	-
	6	Bursa	I	h	15	-	-	-	-
	7	Bursa	I	h	15	-	-	-	-
	8	Bursa	I	h	15	-	-	-	-
Sept11	1	Bursa	I	h	15	-	-	-	-
	2	Bursa	I	h	15	-	-	-	-
	3	Bursa	I	h	15	-	-	-	-
	4	Bursa	I	h	15	-	-	-	-
	5	Bursa	I	h	15	-	-	-	-
	6	Bursa	I	h	15	-	-	-	-
	7	Bursa	I	h	15	-	-	-	-
	8	Balıkesir	J	ı	15	-	-	-	-
	9	Balıkesir	J	ı	12	-	-	-	-
Oct11	1	Balıkesir	J	ı	12	-	-	-	-
	2	Balıkesir	J	ı	12	-	-	-	-
	3	Balıkesir	J	ı	12	-	-	-	-
	4	Balıkesir	K	j	12	-	-	-	-
	5	Balıkesir	L	k	12	-	-	-	-
	6	Balıkesir	M	l	15	-	-	-	-
	7	Balıkesir	M	l	15	-	39	-	-
	8	Balıkesir	M	l	15	-	40	-	-
Nov11	1	Balıkesir	M	m	15	-	-	-	-
	2	Bursa	F	e	12	-	-	-	-
	3	Bursa	F	e	12	-	-	-	-
	4	Bursa	F	e	10	-	-	-	-
	5	Bursa	F	e	15	-	-	-	-
	6	Manisa	C	n	10	-	-	-	-
	7	Manisa	C	n	12	-	-	-	-

Table 1 resume.
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Sampling strategy					Results			
Sampling period	No of samples	City	Supplier	Retailer	No: eggs sampled	Inner positive sample ID		Shell positive sample ID	
						ISO	LCPCR	ISO	LCPCR
Dec11	1	Manisa	C	n	12	-	-	-	-
	2	Manisa	C	n	12	-	-	-	-
	3	Manisa	C	n	12	-	50	-	-
	4	Manisa	C	n	12	-		-	-
	5	Ankara	E	f	15	-	52	-	-
	6	Ankara	E	f	15	-	-	-	-
	7	Ankara	E	f	15	-	-	-	54
Jan12	1	Ankara	E	f	15	-	-	-	-
	2	Ankara	E	f	15	-	56	-	-
	3	Bursa	D	f	15	-	-	-	57
	4	Bursa	D	f	15	-	-	-	58
	5	Bursa	D	f	15	-	-	-	-
	6	Bursa	D	f	15	-	60	-	-
	7	Bursa	D	f	15	-	61	-	-
Feb12	1	Ankara	E	f	15	-	-	-	62
	2	Ankara	E	f	15	-	63	-	-
	3	Ankara	E	f	15	-	64	-	-
	4	Ankara	E	f	15	-	-	-	65
	5	Ankara	E	f	15	-	66	-	-
	6	Bursa	D	f	15	-	-	-	67
	7	Bursa	D	f	15	-	68	-	-
	8	Bursa	D	f	15	-	69	-	-
	9	Bursa	D	f	15	-	70	-	-
	10	Bursa	D	f	15	-	-	-	71
Mar12	1	Balıkesir	N	o	12	-	-	-	72
	2	Balıkesir	N	o	12	73	-	-	-
	3	Balıkesir	N	o	12	-	-	-	-
	4	Balıkesir	N	o	12	-	75	-	-
	5	Balıkesir	N	o	12	-	-	-	76
	6	Balıkesir	O	p	20	-	77	-	-
	7	Balıkesir	O	p	20	78	78	-	-
	8	Balıkesir	O	p	20	-	79	-	-
	9	Balıkesir	O	q	20	80	80	-	-
	10	Balıkesir	O	q	30	-	-	-	81
	11	Balıkesir	O	q	30	-	-	82	82
	12	Balıkesir	O	q	30	83	83	-	-
Apr12	1	Balıkesir	O	r	30	-	84	-	-
	2	Balıkesir	O	r	30	85	85	-	-
	3	Balıkesir	O	r	30	86	-	-	-
	4	Balıkesir	O	r	30	-	87	-	-
	5	Balıkesir	P	s	30	-	-	88	88
	6	Balıkesir	P	s	30	-	-	89	-
	7	Balıkesir	P	s	20	-	-	-	90
	8	Balıkesir	P	s	20	-	91	-	-

Table 1 resume.
Tablo 1 devam.

Sampling strategy					Results			
					Inner positive sample ID		Shell positive sample ID	
Sampling period	No of samples	City	Supplier	Retailer	No: eggs sampled	ISO	LCPCR	ISO	LCPCR
May12	1	Balıkesir	Q	t	30	92	-	92	-
	2	Balıkesir	Q	t	30	-	-	93	93
	3	Balıkesir	Q	t	30	94	-	-	-
	4	Balıkesir	Q	t	30	95	-	-	-
	5	Balıkesir	Q	t	30	96	96	-	-
	6	Bursa	D	f	15	97	97	-	97
	7	Bursa	D	f	15	-	98	-	-
	8	Bursa	D	f	15	-	-	-	99
	9	Bursa	D	f	15	-	100	-	-
	10	Bursa	D	f	15	-	-	-	-
Total	101	5	16	20	1635	11	31	6	18

Table 2. Relative accuracy, sensitivity and specificity results of LCPCR with reference to ISO method
Tablo 2. LCPCR'ın ISO metoduna göre göreceli doğruluk, duyarlılık ve özgünlüğü

Egg part (n)	Reference method		Alternative method		Accuracy (%)	Sensitivity (%)	Specificity (%)
	ISO		LCPCR				
	Positive	Negative	False $_{neg}$	False $_{pos}$			
Inner (101)	6	65	5	25	70.3[a]	72.2	54.5
Shell (101)	4	81	2	14	84.2[b]	85.3	66.7
Whole (101)	10	45	7	39	54.5[c]	53.6	58.8

[a-c] Kappa index values 0.15, 0.27, and 0.27, respectively.
[a-c] Kappa indeks değerleri sırasıyla 0.15, 0.27 ve 0.27'dir.
[a] There was almost no agreement between ISO and PCR.
[a] ISO ve PCR arasında hemen hemen hiçbir uyum bulunmamaktadır.
[b] Fair agreement between methods
[b] Metotlar arasında kayda değer bir uyum bulunmaktadır.
[c] Slight agreement between methods
[c] Metotlar arasında zayıf bir uyum bulunmaktadır.

Serotyping results of the 16 isolates of inner and shell parts revealed that all were SE.

When LCPCR was compared to ISO as the reference method, 5 and 2 false-negative samples were detected in inner and shell parts of the egg samples. The relative accuracy rates were 70.3%, 84.2% for inner egg and shell egg samples, respectively (Table 2), indicating only a fair agreement between ISO and LCPCR on shell samples only. The relative sensitivity and specificity of shell part LCPCRs were comparably higher than those of inner and whole egg samples. The number of false positives in LCPCR resulted in lower agreement between methods, as well as lowering the relative specificity and sensitivity of the test (Table 2).

Discussion and Conclusion

The aim of this study was to determine the presence of *Salmonella* in retail grade A eggs by using both ISO and LCPCR to complement ISO. Our ISO results indicated that 10.9 % of inner and 5.9 % of shell (overall 15.8 %) of the retail eggs harbored *Salmonella*. Our inner part rate is higher than previous studies reported from different parts of the world (4, 8, 12, 19, 28), which have found this rate as 0-7.4 % in retail eggs, contrary to a study by Betancor et al. (2), indicating a considerably higher rate of 35% *Salmonella* from Uruguay. Similarly, there are several previous studies with lower *Salmonella* shell rates as 0-5.3% (4, 12, 24) than ours, two studies with higher rates as 15.8% (21) and 21% (8), and one study by Suresh et al. (28) indicating a slightly higher rate as 6.1% than ours. The differences between the rates we detected with the previously conducted studies can be linked to factors such as: (1) layers: housing, management - production practices, genetic background, *Salmonella* control and vaccination programs used; (2) applied/restricted egg processing practices: washing, sanitizing, oiling, drying, storage temperature conditions, cleaning; (3) region and climate: geographical area,

season; (3) sample: methods used in sampling, method of analysis, sample number, duration of sampling. As indicated by Jones et al. (18), comparison of egg microbiology data on *Salmonella* around the world stays inconclusive and is difficult to compare due to above and related reasons.

The *Salmonella* isolation rate from inner parts of the eggs is higher than the shell rate, contrary to the similar previous studies, which have lower or 0% inner rate compared to higher shell rates (4, 8, 12, 28). This difference could be the result of many contributing factors in those studies and our study, and cannot be extrapolated further.

Our serotyping results revealed that all *Salmonella* isolates belonged to the serovar Enteritidis, similar to the findings of Suresh et al. (28), Adesiyun et al. (1) and Betancor et al. (2), and in contrast to Musgrove et al. (21) and Lee et al. (19).

In this study, we used LCPCR to complement ISO in detecting *Salmonella* from naturally contaminated egg samples, and determined relatively high *Salmonella* detection rates (Overall 46.5%, inner 30.7%, shell 17.8%). The higher positivity in LCPCR had previously been observed in some 'poultry meat-related' LCPCR studies by Bohaychuk et al. (3) and Eyigor et al. (10), and was related factors such as high numbers of non-culturable/dead *Salmonella* cells in the sample, insufficient recovery of sub-lethally injured cells, despite specific enrichment in the culture-based method (3, 9). However, correlating this high LCPCR rate in our egg samples only to the factors mentioned above seemed inconclusive. Therefore, we sought for other reasons for this result, and went through the available product specifications and applicability statement in the kit insert. The sequence-specific Foodproof Salmonella Master Mix Kit designed to amplify 'a highly conserved gene' found in all subgroups of *Salmonella*, was notified as tested for inclusivity with more than 700 strains of *Salmonella* comprising all subspecies, and for exclusivity using 60 species of closely related organisms or organisms occurring in the same habitat. This was satisfactory from the point of specificity, whereas the sensitivity of the kit was slightly high, indicated as 10^4 cfu/ml detection limit in enrichment cultures. This made us think that despite PE and PrE, if/when initial sub-lethally injured/low numbers of cells were present in the eggs, these cells somehow might not have reached the levels in enrichments to compete with non-*Salmonella* flora disturbing selective plate readings, and eventually leading to false negativity in culture. Also, performance of the *Salmonella* culture method (number of positive isolations) was recently reported to depend on other factors, such as to the motility of the *Salmonella* isolate (26, 27), to the choice/appropriateness, and incubation period in selective medium. These may be some of the further contributing factors affecting our relatively lower culture rate, which would still require specific investigations.

The milieu (here egg content/shell) in which the bacterium (here *Salmonella*) resides prior isolation/detection is another equally important issue in defining the appropriateness of the assay kit developed for the detection of a specific pathogen. Therefore, we also checked if this kit was applicable for detecting *Salmonella* from 'naturally contaminated eggs'. Indication of the following note in the applicability statement of the kit insert as: 'the Foodproof Salmonella Detection Kit has an intended use for the rapid detection of *Salmonella* DNA isolated from enrichment cultures prepared by valid methods, such as NordVal with the ISO 6579 method for five food groups including eggs' reassured us that we used a proper kit for our purposes.

In this study, *Salmonella* was detected in 10 egg (inner + shell) samples by both methods, in 6 samples only by ISO compared to 37 positives only by LCPCR (Table 1). In contrast to our high LCPCR positivity, Soria et al. (25) reported that their PCR results were not as good as the culture method results for some *Salmonella* strains, where they compared two culture methods and a PCR assay to detect *Salmonella* in artificially contaminated egg content. They concluded that further studies were required to improve PCR methods' performance parameters and limit of detection compared to culture for the test to find use in poultry and food industry.

Although not entirely relevant, due to incoherence with their scope, we evaluated our LCPCR result with some recently performed study(ies), of none tested their PCR to detect *Salmonella* in potentially naturally contaminated retail eggs. One of these studies was a real-time PCR developed by Chen et al. (5) was used for detecting SE in artificially contaminated foods, including liquid eggs. In another survey study by Gole et al. (12) PCR was utilized to detect the presence of several virulence genes in *Salmonella* they isolated from eggs of commercial laying flocks. A recent study by Lee et al. (19) applied real-time PCR in confirming presumptive *Salmonella* isolates after standard metabolic and biochemical tests. All these studies once more indicate PCR's indispensible advantages in detecting *Salmonella* and SE in complementing culture methods.

To conclude, grade A eggs produced in our country can still pose a risk factor for *Salmonella* in its separate consumption in different forms, and as cross-contamination to other foods in household and catering kitchens. This public health problem as foodborne salmonellosis and outbreaks require fundamental and strict approaches in biosecurity applications in layer farms, with routine *Salmonella* monitoring by reliable detection methods to reduce the presence in retail eggs.

Acknowledgements

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Address for correspondence:
Prof. Dr. Ayşegül Eyigör
Uludağ University,
Faculty of Veterinary Medicine,
Department of Food Hygiene and Technology,
16059, Görükle Campus, Bursa, Turkey
e-mail: aeyigor@uludag.edu.tr