

# Prevalence, Serological Typing and PCR Sensitivity Comparison of *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* spp. Isolated from Raw Chicken Carcasses <sup>[1]</sup>

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## Abstract

Poultry meat is the most popular food products worldwide. *Salmonella* are important foodborne pathogens especially in poultry. Objectives in this study were to determine the presence of *Salmonella* spp. and to detect the incidence of *Salmonella* Typhimurium and *Salmonella* Enteritidis in 100 raw chicken carcasses. Carcasses which were collected from Istanbul (n=100) for the detection of the organism by conventional culture method and confirmed of strains by PCR of DNA using *invA* and *fliC* genes. According to the results, *Salmonella* spp. was determined in 15 (15%) raw chicken carcass samples of 100 total samples analyzed due to both PCR and conventional culture method include serological tests; Four (26.6%) samples were identified as *S. Enteritidis* while 3 (20%) samples were *S. Typhimurium* of 15 total *Salmonella* spp. Sensitivity of PCR procedures for *Salmonella* spp. and *S. Typhimurium* were high and quite specific. However, the sensitivity of the mentioned procedure was very low for *S. Enteritidis*. It is being thought that PCR procedures can be good alternative methods to microbiological analysis procedures for *Salmonella* spp. and *S. Typhimurium* while microbiological analysis procedures have more advantages than PCR procedures for protection of the public health at the detection of *S. Enteritidis*.

**Keywords:** *Salmonella* Enteritidis, *Salmonella* Typhimurium, PCR, Serological typing, Chicken carcass

## Çiğ Tavuk Karkaslarından İzole Edilen *Salmonella* Typhimurium, *Salmonella* Enteritidis ve *Salmonella* spp.'nin Prevalans, Serolojik Tiplendirme ve PCR Hassasiyetinin Karşılaştırılması

## Özet

Kanatlı eti dünyada en popüler gıda ürünlerinden biridir. Bununla birlikte, *Salmonella* özellikle kanatlılarda önemli gıda kaynaklı patojenlerdir. Bu araştırmanın amaçları, *Salmonella* spp. varlığının belirlenmesi ve 100 adet çiğ tavuk karkasında *Salmonella* Typhimurium ve *Salmonella* Enteritidis insidensinin tespit edilmesidir. Ayrıca, izole edilen suşlarda, klasik kültürel metod ve PCR prosedürlerinin etkinliğinin karşılaştırılması da amaçlanmıştır. İstanbul'dan temin edilen karkaslarda (n=100) organizma, klasik kültürel metod ile tespit edilmiş ve tespit edilen edilen suşlar, *invA* and *fliC* genlerinin araştırıldığı PCR analizi ile doğrulanmıştır. Elde edilen bulgulara göre *Salmonella* spp., 100 adet çiğ tavuk karkas örneğinin 15 (%15) adedinde, PCR ve serolojik testleri içeren klasik kültürel metod kullanılarak belirlenmiş ve 15 adet *Salmonella* spp.'nin 4 (%26,6) adedi *S. Enteritidis*, 3 (%20) adedi ise *S. Typhimurium* olarak serotiplendirilmiştir. *Salmonella* spp. ve *S. Typhimurium* tespit için PCR prosedürlerinin duyarlılığı yüksek ve spesifik olarak bulunmuştur. Buna karşın, söz konusu prosedürün duyarlılığı *S. Enteritidis* için oldukça düşük kalmıştır. *Salmonella* spp. ve *S. Typhimurium* teşhisinde PCR prosedürlerinin mikrobiyolojik analiz prosedürlerine göre göre önemli bir alternatif olabileceği düşünülmekte iken, *S. Enteritidis* tespitinde ise halk sağlığının korunması açısından mikrobiyolojik analiz prosedürlerinin PCR prosedürlerine göre daha avantajlı oldukları öngörülmektedir.

**Anahtar sözcükler:** *Salmonella* Enteritidis, *Salmonella* Typhimurium, PCR, Serolojik tiplendirme, Tavuk karkası

## INTRODUCTION

Poultry meat is popular food products worldwide. Several factors such as high level protein and low fat

content and favorable content of fatty acids contribute to the popularity of poultry meat and economics factors are important. Chicken meat is widely used in fast-food establishment and restaurants. Therefore, poultry meat



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comprises about two-thirds of the total production in the world [1]. Poultry meat production was 245,554.1 metric tons in 2012, Turkey. Accordingly, Turkey has been the largest 9<sup>th</sup> poultry meat producer in the world [2].

Foodborne pathogens are evaluated as serious risk factors from producing processes up to consuming for public health. Salmonellosis is one of the most common and widely distributed foodborne diseases and is caused by the bacteria *Salmonella*. It is estimated that tens of millions of human cases occur worldwide every year and the disease results in more than hundred thousand deaths [3]. Furthermore, one of the commonest causes of salmonellosis reported humans has been through the handling of raw carcasses and products, together with the consumption of undercooked poultry meat [4]. The global increase in chicken consumption stimulated by its high protein content and its accessible price has drawn the attention of producers, researchers and authorities to the necessary of controlling *Salmonella* contamination, principally during the various stages commercial production chains [5].

The prevalence of *Salmonella* spp. in chicken meat has been studied in many countries [1,5-9] including Turkey [10-13]. However, very little statistical data on *Salmonella* serotypes and infections collected from individual studies are available in Turkey [11]. *Salmonella* Typhimurium (*S. Typhimurium*) and *Salmonella* Enteritidis (*S. Enteritidis*) are the most predominant isolated organisms associated with the consumption of contaminated poultry, pork and beef meats and products [7]. Furthermore, the two most important serotypes of salmonellosis transmitted from animals to humans in most parts of the world [3]. Similarly, *S. Typhimurium* and *S. Enteritidis* are the most common serotypes isolated from humans in Turkey [14].

Many techniques, i.e. conventional culture, molecular biological and immunological, are being used for the detection of *Salmonella* spp. [10]. Culture based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity. Depending on the approach, standard culture methods typically require 5-7 days to obtain a result as they rely on the ability of *Salmonella* to multiply to visible colonies, which can then be characterized by performing additional biochemical and or serological tests [15]. Polymerase Chain Reaction (PCR) is a simple, rapid, very specific, and relatively inexpensive technique [5]. Currently, the use of PCR being one of the most promising approaches for the detection of *Salmonella* serotypes [5,9].

The objectives in this study were (1) to explore the prevalence of *Salmonella* spp. in the 100 raw chicken carcass samples obtained from butchers and supermarkets in Istanbul (2) to determine the incidence of *S. Typhimurium* and *S. Enteritidis* which are the two most dangerous

strains for the public health among *Salmonella* spp. with PCR procedures (3) to compare the effectiveness of the PCR and conventional microbiological methods for the mentioned pathogens.

## MATERIAL and METHODS

### Sampling

One hundred raw chicken carcasses were collected between from different sales points (supermarkets (n=50) and butchers (n=50) in Istanbul, Turkey. Fifty samples were collected from European side (supermarkets (n=25) and butchers (n=25) and the other 50 samples were collected from Asian side of Istanbul (supermarkets (n=25) and butchers (n=25)). Random sampling method was used during sampling period and middle class supermarkets and district butchers were preferred.

All the collected samples were consisted of raw chicken carcasses, the supermarket samples were packaged while the butcher samples were purchased open. Samples were transported to the laboratory after being collected in a thermobox under cold chain (+4°C) and microbiological analyses were carried out immediately.

### Isolation and Identification

Samples of skin and muscle, amounting 25 g, taken from multiple parts of chicken carcasses were homogenized in a stomacher (Interscience, Saint Nom, France) with 225 ml with buffered peptone water (Oxoid CM 1049) for non-selective enrichment. After incubation at 37°C for 24 h, 0.1 ml was inoculated in 10 ml Rappaport Vassiliadis Soy (RVS) Broth (Oxoid CM 866) for selective enrichment and incubated at 42°C for 24 h. After selective enrichment procedure, a loopful of broth was streaked on Brilliant Green Phenol Red Lactose Sucrose (BPLS) Agar (Oxoid CM 263) and Xylose Lysine Desoxycholate (XLD) Agar (Oxoid CM 469) parallel and incubated at 37°C for 24 h. After the detection of presumptive colonies on agars, the colonies sub-cultured to Nutrient Agar (NA; Oxoid CM 003) were confirmed as *Salmonella* by inoculation on Triple Sugar Iron Agar (TSIA) (Oxoid CM 277), urea broth (Oxoid CM 071) and Lysine Iron Agar (LIA) (Oxoid CM 381), followed by incubation of the tubes at 35-37°C for 24-48 h. Finally, API 20E (bioMerieux® SA, Marcy l'Etoile, France) kits were used according to the manufacturer's directions for the determination of *Salmonella* spp. to the species level.

### Serological Identification

Serotype identification of the 15 positive *Salmonella* strains performed according to the White-Kaufmann-Le Minor scheme with lam agglutination and serum neutralization tests [16]. According to agglutination tests, commercial phase 1 and phase 2 antisera and *Salmonella* somatic group (O) and flagella group (H) antigens provided by Difco (Becton Dickinson Co., New Jersey, USA) were used.

### DNA Extraction

DNA extraction procedure was adapted from Oliveira et al.<sup>[17]</sup>. Bacteria were cultured on TSB for overnight at 37°C. 1 ml aliquot of broth was centrifuged at 2.000 x g for 4 min and the bacterial pellet resuspended in TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) containing lysozyme (Sigma 7651) and incubated at 4°C for 30 min, after which 25 µl SDS and Proteinase K (20 mg/ml, Merck 124568) were added and incubated at 55°C for 30 min. Then, 500 µl phenol-chloroform pH 8.0 was added and DNA was precipitated with sodium acetate and cooled isopropanol and centrifuged 16.000 x g for 10 min at 4°C, following supernatant was removed and the pellet washed with 1 ml 80% cooled ethanol (Sigma 459844), the pellet being resuspended in 50 µl of TE and stored at -20°C.

### PCR Analysis

PCR mix was as follows (final 25 µl); 2 µl DNA samples, 2.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 8.0, 5 mM KCl (0.2 mM from each nucleotide), each primer (Metabion International, Martinsried, Germany) 0.8 pmol/ml, 1 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania). The primer sequences used in PCR analysis are shown in [Table 1](#). Initial denaturation heat was at 94°C for 5 min. Then the heat treatments, 1 sec at 94°C, 1 sec at 55°C, and 21 sec at 72°C for extension were applied. After 35 cycles, the procedure was completed with 7 min at 72°C heat treatment for last elongation. Amplication products were analyzed in 1.2% (w/v) agarose gel containing 5 µl safe view (Abm, Richmond, Canada).

### PCR Specification, Calculation of Specificity and Sensitivity

The relative sensitivities of PCR procedures are described as the rate of obtained PCR products to isolated cultures with reference methods <sup>[21]</sup>. Relative sensitivity (SE) and relative specific (SP) degrees of the PCR procedures applied in our study were calculated by using the formulas indicated below:

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

PA: Positive Agreement

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

$$SP = \frac{NA \text{ value of PCR and reference culture methods}}{N \times 100}$$

NA: Negative Agreement

N: Number of negative samples obtained with reference isolation/identification methods <sup>[22]</sup>.

For determination of PCR sensitivity, reference *S. Enteritidis*, and *S. Typhimurium* strains were serially diluted with 0.1% peptone water (Oxoid CM 009) up to 10<sup>-9</sup> concentration level (1-10 kob ml<sup>-1</sup>) so that 5 replication. Grown strains were evaluated as 10<sup>-9</sup> dilutions of that *Salmonella* serotypes and the strains were passage to NA, including 7 grams/liter yeast extract (Oxoid CM 019). Additionally, a non-*Salmonella* mixture consisted of 5 different non-*Salmonella* strains were treated with 0.1% peptone water up to 10<sup>-4</sup> dilution concentration. For each *Salmonella* dilution, 1 ml of non-*Salmonella* mixture were added to the tubes that included *Salmonella* serotypes and the bacterial mixtures were incubated at 37°C for 24 h. After the incubation period, each mixture was passed to RVS broth of 10 ml. Then, a last incubation at 37°C 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

One hundred samples of retail chicken carcasses were analyzed for *Salmonella* spp. and the prevalence of *Salmonella* spp. was detected 15% in chicken carcass samples. Seven (46.6%) chicken carcasses samples obtained from Anatolian side and 8 (53.3%) chicken carcasses samples obtained from European side in Istanbul of the total 15 *Salmonella* positive carcass samples. [Table 2](#) shows that the *Salmonella* contamination rates were 73.3% (11/15) and 26.6% (4/15) in butcher and supermarket originated chicken carcasses, respectively.

*Salmonella* spp., *S. Enteritidis* and *S. Typhimurium* was determined at rate 53.3% (8/15), 26.6% (4/15), and 20% (3/15) of *Salmonella* positive samples, respectively. *S. Enteritidis* isolated only one (25%) supermarkets originated chicken carcass samples from Asian side, Istanbul. On the other hand, all the *S. Typhimurium* positive (n=3) chicken samples originated from butchers (one (33.3%) samples

**Table 1.** The properties of primer sequences designed according to different *Salmonella* serotypes

**Tablo 1.** Farklı *Salmonella* serotiplerine göre dizayn edilen primer dizilerinin özellikleri

Gene/bp	Virulence Factor	Primers 5' – 3'	Target Microorganism	Reference
<i>invA</i> /284	Invasion	F-GTGAAATTATCGCCACGTTCCGGGCAA R-TCATCGCACCGTCAAAGGAACC	<i>Salmonella</i> spp.	[18]
<i>fliC</i> /620	Flagella	F-CGGTGTTGCCAGGTTGGTAAT R-ACTGGTAAAGATGGCT	<i>S. Typhimurium</i>	[19]
<i>sefA</i> /488	Fimbria	F-GATACTGCTGAACGTAGAAGG R-GCGTAAATCAGCATCTGCAGTAGC	<i>S. Enteritidis</i>	[20]

**Table 2.** Results of the *Salmonella* positive samples**Tablo 2.** *Salmonella* pozitif örnekler için sonuçlar

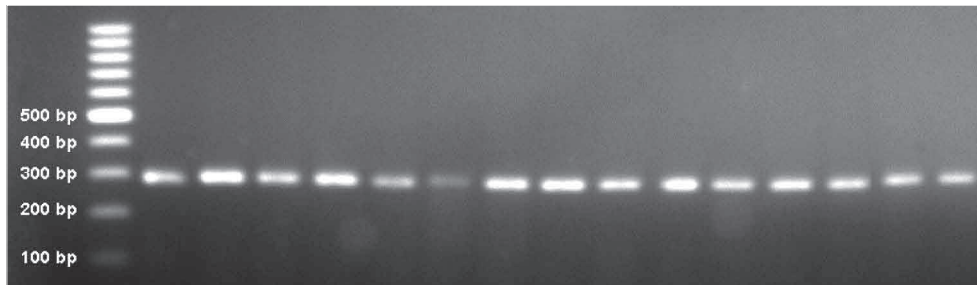
Species	Sales Point/ *A or E	Conventional Culture Method	PCR Verification
<i>Salmonella</i> spp.	Supermarket (A)	+	+
<i>Salmonella</i> spp.	Supermarket (A)	+	+
<i>Salmonella</i> spp.	Supermarket (E)	+	+
<i>Salmonella</i> spp.	Butcher (A)	+	+
<i>Salmonella</i> spp.	Butcher (E)	+	+
<i>Salmonella</i> spp.	Butcher(A)	+	+
<i>Salmonella</i> spp.	Butcher (E)	+	+
<i>Salmonella</i> spp.	Butcher (E)	+	+
<i>S. Typhimurium</i>	Butcher (A)	+	+
<i>S. Typhimurium</i>	Butcher (E)	+	+
<i>S. Typhimurium</i>	Butcher (E)	+	+
<i>S. Enteritidis</i>	Butcher (E)	+	+
<i>S. Enteritidis</i>	Supermarket (A)	+	+
<i>S. Enteritidis</i>	Butcher (E)	+	+
<i>S. Enteritidis</i>	Butcher (A)	+	+

\*A or E: Anatolian side or European side of Istanbul

For determining PCR sensitivity and detection limits, different concentrations of *S. Enteritidis* and *S. Typhimurium* were prepared, and the minimal detection of concentration levels were tried to determine for the mentioned strains. The minimal PCR detection limit was 8 cells for *S. Typhimurium* and  $1.8 \times 10^3$  cells for *S. Enteritidis*, respectively.

## DISCUSSION

*Salmonella* spp. continues to be a leading cause of foodborne illness. Raw poultry, meats, and meat derived products are important vehicles of human salmonellosis; however, increasingly, illnesses are associated with the consumption of fresh products and dry food products [23]. Chicken and chicken products are widely known to be an important reservoir for *Salmonella*, and they have been ascribed as vehicles of salmonellosis [6-8]. In this study the incidence of *Salmonella* in raw chicken carcasses was 15%. Similar results reported by Zahraei Salehi et al. [9] who isolated *Salmonella* spp. at rate of 15.6% (Iran), and Todd [24] reported *Salmonella* prevalence of 13.3% in retail chicken in Ethiopia. Another study conducted by Alali



**Fig 1.** Agarose gel electrophoresis of the amplified products of PCR to determine strains of the *Salmonella* spp. (*invA* gene, 284 bp). Lane 1: 100 bp marker (Thermoscientific), Lane 2-16: positive *Salmonella* strains

**Şekil 1.** Tespit edilen *Salmonella* spp. (*invA* geni, 284 bp) suşlarının amplifiye PCR ürünlerinin agaroz jel elektroforezi. Sıra 1: 100 bp marker (Thermoscientific), Sıra 2-16: Pozitif *Salmonella* suşları

in Asian side and two (66.7%) samples from European side, Istanbul. Additionally, *Salmonella* spp. contamination rate were 37.5% (3/8) and 62.5% (5/8) in supermarket and butcher originated chicken carcasses, respectively. *Salmonella* spp. isolated 33.3% (1/3) supermarkets originated chicken carcass samples from European side, Istanbul. Additionally, 3 (60%) positive *Salmonella* spp. butchers originated chicken carcass samples from European side, Istanbul.

All the strains were isolated by conventional culture method and confirmed by PCR (Table 2). At the PCR optimization process, the usage of magnesium chloride at a concentration of 2 mM gave the best band results for *sefA* genes. For the other two genes (*invA* and *fliC* target genes) any significant differences was observed at the amplification period. At the end of the PCR process, 284 bp amplification products were obtained for the *invA* targets for all the *Salmonella* spp. positive samples (Fig. 1).

et al. [25] in Russia Federation showed the incidence of *Salmonella* spp. in retail chicken to be 27%. Our results of contamination rates with *Salmonella* lower than those observed by das Chagas et al. [5] 94% (Brazil), Capita et al. [6] 55% (Spain), Yang et al. [8] 52.2% (China), Abd El-Aziz [1] 44% (Egypt) and Uyttendaele et al. [7] 37% (Belgium). Not much more reference has been available on the presence of *Salmonella* in meat from Turkey, few researchers reported prevalence as low as 10% [10,11,13]. The differences in these contamination percentages are probably related to numerous factors, including the origin of the chicken lots, the sampling methods, microbiological analysis methods, the hygiene-sanitary conditions in the abattoirs, and cross-contamination that occurred during plucking, washing, cooling and wrapping [25].

As shown Table 2, the contamination rate in butcher's carcasses (73.3%) was higher than in supermarket carcasses

(26.6%). Additionally, the contamination rate of *Salmonella* was higher (53.3%) chicken carcasses originated in European side, Istanbul. Similarly, Plummer et al.<sup>[26]</sup> detected a lower number of *Salmonella* supermarkets originated carcasses (18.6%), than from shops (24.5%). Conversely, Capita et al.<sup>[6]</sup> was found the *Salmonella* prevalence 75% and 25% in supermarket carcasses and poultry shops carcasses, respectively. The differences can be related continue temperature control of refrigerators or ambient temperature in supermarkets by responsible persons.

Two different serotypes, *S. Enteritidis* and *S. Typhimurium*, were found as seen from [Table 2](#). *S. Enteritidis* was the most commonly isolated serotype (26.6%) in our study. The high percentage of *S. Enteritidis* in chicken samples with the results obtained by other researchers<sup>[5,7,11,26]</sup>. Additionally, 3 (20%) samples were evaluated as positive *S. Typhimurium*. One of the most important characteristics of *S. Typhimurium* in meat and its products, is the tolerance to acidic media. The elevated presence of this serotype in chicken products agrees with *S. Enteritidis* being the predominant serotype associated with outbreaks due the consumption of eggs<sup>[6]</sup>. Accordingly, *S. Enteritidis*, *S. Typhimurium*, *S. Paratyphi B* and *S. Typhi* are the most common serotypes isolated from humans<sup>[14]</sup> and poultry<sup>[27]</sup> in Turkey.

In current study, *invA* gene used for the detection for *Salmonella*. Similarly, Rahn et al.<sup>[18]</sup> indicated that different *Salmonella* serotype have *invA* gene. This gene is recognized internationally as a standard for detecting the genus *Salmonella*, and its amplification has been used by many researchers to detect contamination in chicken carcasses<sup>[5,9,17,20]</sup>. For *S. Typhimurium*, DNA fragments that had, *fliC* target genes were amplified (620 bp) while, DNA fragments that had *sefA/invA* target genes were amplified for *S. Enteritidis* (488 bp) ([Table 2](#)). Similar to the our study, das Chagas et al.<sup>[5]</sup>, Olivieara et al.<sup>[17]</sup>, and Doran et al.<sup>[20]</sup>, were used *invA* gene, *fliC* and *sefA* gene for the detection of *Salmonella* spp., *S. Typhimurium* and *S. Enteritidis*, respectively. The *fliC* gene is responsible for the expression of a protein known as flagellin in *Salmonella* spp. the *sefA* gene codes for the fimbrial protein SEF14 that has unique specificity to *S. Enteritidis* and its amplification can be used identify this serotype<sup>[28]</sup>.

PCR sensitivity was determined as 8 cells for *S. Typhimurium* while mentioned value was 300 cells according to Rahn et al.<sup>[18]</sup>. By using conventional microbiological methods, to isolate one or several target microorganisms, especially for *Salmonella* spp. from a food sample may be very hard. Accuracy and sensitivity of the dilution, long time of isolation procedure, a requirement of professional hand practices, and qualities of the media that is used are the main factors that complicate the isolation and identification procedures of *Salmonella* spp.<sup>[29]</sup>. 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 *Salmonella* spp.. The PCR procedures that we used gave quite specific results for *S. Typhimurium* at a serotype level. By specification of target primers, *S. Typhimurium* positive evaluated samples by microbiological methods exactly matched with the PCR results while the other *Salmonella* strains positive results did not. Moreover, the results can be obtained average 5 days earlier by PCR procedures when compared with conventional microbiological methods.

According to the results, PCR sensitivity was about  $1.8 \times 10^3$  cells level for *S. Enteritidis* and this rate was quite low when the PCR procedure was compared with conventional microbiological methods, because, we were able to identify *S. Enteritidis* colonies from  $1 \times 10^1$  colonies concentration level by using microbiological procedures. Doran et al.<sup>[20]</sup> declared that they could identify *S. Enteritidis* from 10 cells of concentration level while Woodward and Kirwan<sup>[30]</sup> could identify the same microorganism from 4 cells. The reason explained above, may be the cause of low degree sensitivity of PCR for *S. Enteritidis*. Another possible cause may also be the hybridization of mobile DNA fragments through polymorphic proteins in spite of *sefA* primer is specific for *S. Enteritidis*. It is thought that detailed genomic DNA studies would help to clarify genomic and biochemical mechanisms of *Salmonella* strains.

The prevalence of *Salmonella* spp. was relatively high from raw chicken carcasses in Istanbul. The most predominant serotypes are *S. Enteritidis* and *S. Typhimurium* in chicken samples. On the other hand, PCR results were quite specific and sensitive for *Salmonella* spp. and *S. Typhimurium* in our study. For the identification of mentioned two strains, PCR procedures may be a good alternative to microbiological isolation and identification methods. However, results about *S. Enteritidis* were not effective as *Salmonella* spp. and *S. Typhimurium*. Because of the reason explained, using microbiological identification methods for *S. Enteritidis*, would be more effective than PCR procedures for diagnosis and public health.

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