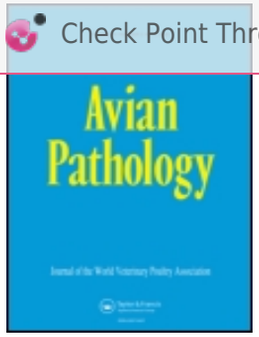

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Salmonella profile in chickens determined by real-time polymerase chain reaction and bacteriology from years 2000 to 2003 in Turkey

Aysegul Eyigor[1], Gulsen Goncagul[2], Elcin Gunaydin[3] and K. Tayfun Carli[3]*

[1]*Department of Food Hygiene and Technology,* [3]*Department of Microbiology, Faculty of Veterinary Medicine, and* [2]*Yenisehir Ibrahim Orhan Vocational High School, Uludag University, Gorukle Kampusu, 16059 Bursa, Turkey*

From years 2000 to 2003, *Salmonella* was investigated from a total of 1785 samples comprised of chicken intestinal samples, cloacal swabs, drag swabs, litter samples and chick dust samples collected from 191 poultry breeding flocks belonging to 15 different chicken breeding stock companies in the Marmara region, Turkey by a SYBR green-based real-time polymerase chain reaction (SGBRT-PCR), by a probe-specific real-time polymerase chain reaction (PSRT-PCR) and by standardized bacteriology as described in the manual of National Poultry Improvement Plan and Auxillary Provisions, United States Department of Agriculture. Between January 2000 and July 2001, *Salmonella* was detected at the rates of 5.87% and 4.10% out of a total of 1242 samples by SGBRT-PCR and bacteriology, respectively. From July 2001 until December 2003, *Salmonella* was found at rates of 11.42% and 5.52% from a total of 543 samples by PSRT-PCR and bacteriology, respectively. The dominant *Salmonella* serovar was determined as *Salmonella enterica* subsp. *enterica* Serovar Enteritidis (*S*. Enteritidis), while serogroup C1 and C2 in 2001 and serogroup E1 in 2002 were isolated as additional serovars. As a conclusion, *S*. Enteritidis seems to be the major problem in poultry breeding flocks in Turkey, and both of the real-time polymerase chain reaction methods were found more sensitive than standard bacteriology for the detection of *Salmonella* from poultry samples.

Introduction

In studies performed in the 1980s, *Salmonella enterica* subsp. *enterica* Serovar Gallinarum (*S*. Gallinarum) and *Salmonella enterica* subsp. *enterica* Serovar Typhimurium (*S*. Typhimurium) were reported as the dominant serovars in chicken flocks in Turkey, while by the beginning of 1990s *Salmonella enterica* subsp. *enterica* Serovar Enteritidis (*S*. Enteritidis) started to replace these aforementioned serovars (Carli, 1990; Carli *et al*., 2001a). For example, in our previous report in 2001, out of 814 ileocecal intestinal samples that were examined by bacteriology, 151 (18.6%) samples were found positive for *Salmonella*. The serovar breakdown for these positive samples were reported as 81.5% for *S*. Enteritidis, 10.1% for *Salmonella enterica* subsp. *enterica* serovar Thompson, 7.6% for *Salmonella enterica* subsp. *enterica* serovar Agona, and 0.8% for *Salmonella enterica* subsp. *enterica* serovar Sarajane in the same study (Carli *et al*., 2001a). Currently, we are studying the prevalence of *Salmonella* in retail chicken and turkey meats—and preliminary data indicate that there is high contamination rate of *S*. Enteritidis in these types of meats, which pose an important risk for human health (unpublished data).

Since *Salmonella* detection by bacteriological methods usually requires 5 to 11 days (United States Department of Agriculture Animal and Plant Health Inspection Service [USDA, NPIP], 1996), and samples with low numbers of *Salmonella* cells, usually seen in subclinically infected chickens, may give false-negative results (Fricker, 1987), efforts have been made to reduce the time required and to increase the sensitivity of the methods to detect *Salmonella* serovars in poultry samples (Mandrell & Wachtel, 1999). The polymerase chain reaction (PCR) with preincubation in an enrichment broth has been performed for human (Chiu & Ou, 1996; Luk *et al*., 1997; Lin & Tsen, 1999), animal (Stone *et al*., 1994; Cohen *et al*., 1996), faecal, and food (Aabo *et al*., 1995; Bennett *et al*., 1998) samples. PCRs with preincubations have been found useful and more rapid method because preincubation increases the number of viable *Salmonella* in the sample, and therefore increases the sensitivity of the PCR (Chiu & Ou, 1996; Gouws *et al*., 1998). In addition to the *Salmonella*-PCR studies mentioned, we applied two real-time PCR methods to tetrathionate broth (TTB) enrichment culture of a standard *Salmonella* isolation method from poultry. Thus, we were able to monitor and determine *Salmonella*-infected flocks by reliable and rapid primary screening procedures (Eyigor *et al*., 2002; Eyigor & Carli, 2003).

*To whom correspondence should be addressed. Tel: + 90 224 4429200, ext. 137. Fax: + 90 224 4428025. E-mail: tayfun@uludag.edu.tr
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The main purpose of this study was to investigate the *Salmonella* load in chicken breeding flocks in Turkey, and to test the potential use of our real-time PCR methods (Eyigor *et al*., 2002; Eyigor & Carli, 2003) in comparison with bacteriology (USDA, NPIP, 1996) for the detection of *Salmonella* from poultry samples.

Materials and Methods

Salmonella control strain. *S.* Enteritidis 64K, kindly obtained from Prof. Poppoff (Institute of Pasteur, France), was used in real-time PCRs and in bacteriology as a positive control strain.

Clinical samples. A total of 1785 samples of broiler breeding flocks, including chicken intestinal samples, cloacal swabs, drag swabs, litter samples and chick dust samples, were used. The total number of poultry breeding flocks belonging to 15 different chicken breeding stock companies was 191. A range of one to 40 chicken intestinal samples, taken randomly from 167 of 191 flocks, where each sample had five ileocecal junctions belonging to five individual chickens, were submitted to the laboratory in 5 ml TTBs (Oxoid 235780) in an ice box within 2 h after sampling. Cloacal swabs were sampled from 24 of 191 flocks, and each cloacal swab belonged to one adult chicken. Drag swabs were sampled from 106 out of 191 flocks, where each drag swab represented one flock. Drag swabs were then put into 50 ml TTB, and transferred to the laboratory. A total of 58 litter samples, taken from nine different flocks, where the number of samples ranged from one to 11, were tested as individual samples. Chick dust samples were taken from three individual flocks and were treated similar to litter samples.

Salmonella bacteriology. One gram from each homogenized intestinal sample and each cloacal swab was inoculated into 10 ml TTB. Each drag swab in 50 ml TTB was transferred into 200 ml TTB upon arrival at the laboratory. One gram from each litter and chick dust sample was inoculated into 10 ml TTB. All of these TTBs were incubated at 37°C for 18 h. Twenty microlitres of the TTB culture was then streaked onto a Xylose Lysine Tergitol 4 Agar (Difco 0234-17-9) plate and was incubated at 37°C for 24 h. After incubation, *Salmonella*-suspect colonies were selected, biotyped and serotyped by standard techniques (USDA, NPIP, 1996). Briefly, one suspect colony was inoculated into Triple Sugar Iron Agar (Difco 0265-17-1), and Lysine Iron Agar (Difco 0849-17-6). Colonies revealing positive reaction for *Salmonella* were serogrouped by the following antisera: *Salmonella* "O" Antiserum Poly A (Difco 2534-47-6), *Salmonella* "O" Antiserum Poly B (Difco 2535-47-5), *Salmonella* "O" Antiserum Factor 1 (Difco 2658-47-6), *Salmonella* "O" Antiserum Factor 4 (Difco 2659-47-5), *Salmonella* "O" Antiserum Factor 5 (Difco 2660-47-2), *Salmonella* "O" Antiserum Factor 9 (Difco 2818-47-3), *Salmonella* "O" Antiserum Factor 12 (Difco 2779-47-0), *Salmonella* "O" Antiserum Factor 14 (Difco 2661-47-1), *Salmonella* "O" Antiserum Group C1 Factors 6, 7 (Difco 2949-47-5), *Salmonella* "O" Antiserum Group C2 Factors 6, 8 (Difco 2950-47-1), and *Salmonella* "O" Antiserum Group E1 Factors 3, 10 (Difco 2952-47-9). TTBs of the negative samples were allowed to incubate at 20°C for 5 days as delayed secondary enrichment, and for each sample 100 l of delayed secondary enrichment culture was transferred to a new TTB and was incubated at 37°C for 18 h. After incubation, 20 µl TTB culture was inoculated onto Xylose Lysine Tergitol 4 Agar plate and was incubated at 37°C for 24 h. Suspect colonies were biotyped and serotyped as already described.

Template preparation for real-time PCRs. TTB primary enrichment cultures of the *Salmonella* control strain *S.* Enteritidis 64K and of the clinical samples were used for template DNA preparations for PCR as described by Carli *et al*. (2001b).

Primers and probes for real-time PCR. *Salmonella invA* gene-specific primers described by Rahn *et al*. (1992) were used in a SYBR green-based real-time PCR (SGBRT-PCR). In the probe-specific real-time PCR (PSRT-PCR), two fluorescence-labelled hybridization probes were designed and used together with the aforementioned primers (Eyigor & Carli, 2003).

SGBRT-PCR. We used the LightCycler PCR (Roche Diagnostics) methodology for this purpose, as described in Eyigor *et al*. (2002). For this assay, 2 µl of the template DNA from each sample or from the positive control was added into an 18 µl PCR mixture in a reaction capillary. Two microlitres of deionized water was used in a 20 µl reaction as a no-target control. The reaction mixture optimized for SGBRT-PCR contained the following: 2 µl of 10 x concentration of LightCycler-DNA Master SYBR Green ready-to-use reaction mix for PCR (Taq DNA polymerase, reaction buffer, dNTP mix [with dUTP instead of d TTP], SYBR Green I dye, and 10 mM $MgCl_2$), 2.4 µl of 25 mM $MgCl_2$, 1 µl of each 100 µM primer, 2 µl template and 11.6 µl deionized water. The amplification protocol was one cycle at 94°C for 30 sec, followed by denaturation at 95°C, 5 sec of annealing at 55°C, and 10 sec of primer extension at 72°C for 40 cycles. The temperature transition rate was 20°C/sec. Fluorescence was measured automatically at the end of each extension step.

PSRT-PCR. We used the LightCycler PCR methodology for this purpose (Eyigor & Carli, 2003). Two microlitres from the sample template, *Salmonella* Enteritidis 64K culture template, or deionized water was added into an 18 µl PCR mixture as unknown, positive control, or negative (no-target) control, respectively. The reaction mixture optimized for PSRT-PCR contained the following: 2 µl of 10 x concentration PC2 buffer (Ab Peptides, St Louis, Missouri, USA), 2 µl deoxynucleoside triphosphate mix (2 mM each), 1 µl of each primer and probe (5 pmol/µl each), 1 µl bovine serum albumin (5 ng/µl), 0.2 µl *Taq* DNA polymerase (5 U/µl; Promega, Madison, Wisconsin, USA), 2 µl template DNA, and 8.8 µl deionized water. The amplification protocol included the initial denaturation step at 95°C for 30 sec and 40 cycles of denaturation at 95°C for 0 sec, annealing at 55°C for 5 sec, and 10 sec of primer extension at 72°C. Temperature transition rate was 20°C/sec. Fluorescence (F3) values of each sample were measured at 705 nm automatically at the end of each annealing step.

Statistical analysis. The differences in *Salmonella* detection rates by PCR (either by SGBRT-PCR or by PSRT-PCR) and bacteriology were examined by the McNemar Test (Graph Pad Instat™ Copyright© 1990–1993 Graphpad software, V2.02, Dr Granger LSU Medical Center 931 521S) for each individual year.

Results

Overall, *S.* Enteritidis was observed as the dominant serovar in each period of time of this study. Besides this serovar, in 2000, two *Salmonella* isolates serogrouped as C1 and C2 were recovered from two different drag swabs belonging to different flocks of two individual companies. In 2002, one isolate from intestinal samples was identified as serogroup E1 in one flock.

Between January 2000 and July 2001, 66 (8.25%) out of 800 intestinal samples, five (1.51%) out of 331 cloacal swabs, and two (4.0%) out of 50 drag swabs were found to harbour *Salmonella* by SGBRT-PCR, whereas bacteriological examinations revealed *Salmonella* detection as 47 (5.87%), two (0.60%), and two (4.0%) from the same samples, respectively (Table 1).

In the term between July 2001 and December 2001, 16 (11.76%) and eight (5.88%) intestinal samples were determined as positive for *Salmonella* by PSRT-PCR and bacteriology, respectively. There was no *Salmonella* detection either by PSRT-PCR or bacteriology from any of the 65 cloacal swabs examined (Table 2).

Between January 2002 and December 2002, none of the seven cloacal swabs tested were found positive for *Salmonella*. From a total of 157 intestinal samples examined in this period, PSRT-PCR and bacteriology detection rates for *Salmonella* were found to be 10.82% and 2.54%, respectively (Table 2).

Table 1. *Salmonella detection rates between January 2000 and July 2001*

Sample type (*n*)	Method used		Serovar/serogroup of the isolate
	SGBRT-PCR (%)	Bacteriology (%)	
Intestinal samples (800)	66 (8.25)	47 (5.87)	47 *S*. Enteritidis
Cloacal swabs (331)	5 (1.51)	2 (0.60)	2 *S*. Enteritidis
Drag swabs (50)	2 (4)	2 (4)	1 serogroup C1, 1 serogroup C2
Litter samples (58)	0 (0)	0 (0)	
Chick dust[a] (3)	0 (0)	0 (0)	
Total (1242)	73[A] (5.87)	51[B] (4.10)	

[a]Dust occuring in the hatchery during and after the hatchery processes.
[A,B]Different uppercase superscripts indicate significant difference ($P < 0.001$; McNemar's test).

In the year 2003, *Salmonella* was detected in 9.83% and 30.35% of the 122 intestinal and 56 drag swabs, respectively by PSRT-PCR. Bacteriology detection rates from the same samples were 11.47% and 7.14%, respectively (Table 2).

The difference between PCR (either by SGBRT-PCR or by PSRT-PCR) and bacteriology for the detection of *Salmonella* was found statistically significant ($P < 0.001$), regardless of the year.

Discussion

The *Salmonella* detection rate by bacteriology versus SGBRT-PCR was 51 (4.10%) to 73 (5.87%) out of a total of 1242 clinical samples between the terms of January 2000 and July 2001. These data indicate that SGBRT-PCR had higher *Salmonella* detection capacity over the bacteriology used, as supported by our previous study (Eyigor *et al*., 2002). Possible reasons for the lower detection rate in bacteriology can be as follows: salmonellae found in natural samples may show atypical biochemical profiles and may not be detected in bacteriology (Bennett *et al*., 1998), and *Salmonella* cells may be present in a viable but non-culturable status (Knight *et al*., 1990).

By July 2001, we initiated *Salmonella* detection by our optimized PSRT-PCR to confirm reliably the PCR product specificity by the use of specifically designed probes and by analysing the melting temperatures of these probes after the PCR (Eyigor & Carli, 2003). Comparisons between bacteriology and PCR results for the years 2002 and 2003 also revealed the higher *Salmonella* detection capacity of PSRT-PCR over that of bacteriology (Table 2). However, two intestinal samples were found to be negative in PCR, while bacteriology results for these samples were positive (Table 2). We consider that these two samples could have had considerably high amount of inhibitory substances for the PCR, which could possibly not have been eluted or eliminated either by TTB primary enrichment step of the bacteriology or at the DNA template preparation stage as previously reported (Kongmuang *et al*., 1994; Stone *et al*., 1994; Chiu & Ou, 1996).

Conventional methods to identify *Salmonella,* such as bacteriology, usually require 5 to 11 days (USDA, NPIP, 1996; Andrews & Hammack, 1998). In order to overcome this time problem, we have been working on the development of rapid PCR techniques (Carli *et al*., 2001b; Eyigor *et al*. 2002, Eyigor & Carli, 2003), and have been implementing these techniques to the poultry sector since 1999. By the PCR techniques (SGBRT-PCR and PSRT-PCR) used in this study, poultry premises were rapidly informed about the presence of *Salmonella* in their flocks. After this initial information, detailed bacteriology results, including serotype and antibiotic sensitivity results of the *Salmonella* isolates, were also given to the companies. According to these results, companies could pursue the serotype-specific (Cooper *et al*., 1994; Woodward *et al*., 2002) and non-specific (Nurmi & Rantala, 1973; Fernandez *et al*., 2000)

Table 2. *Salmonella detection rates between August 2001 and December 2003*

Sample type (*n*)	Method used		Serovar/serogroup of the isolate
	PSRT-PCR (%)	Bacteriology (%)	
August 2001 to December 2001			
Intestinal samples (136)	16 (11.76)	8 (5.88)	8 *S*. Enteritidis
Cloacal swabs (65)	0 (0)	0 (0)	
Subtotal (201)	16[A] (7.96)	8[B] (3.98)	
January 2002 to December 2002			
Intestinal samples (157)	17 (10.82)	4 (2.54)	1 Serogroup E1, 3 *S*. Enteritidis
Cloacal swabs (7)	0 (0)	0 (0)	
Subtotal (164)	17[A] (10.36)	4[B] (2.43)	
January 2003 to December 2003			
Intestinal samples (122)	12 (9.83)	14 (11.47)	14 *S*. Enteritidis
Drag swabs (56)	17 (30.35)	4 (7.14)	4 *S*. Enteritidis
Subtotal (178)	29[A] (16.29)	18[B] (10.11)	
Total (543)	62 (11.42)	30 (5.52)	

[A,B]Different uppercase superscripts indicate significant difference ($P < 0.001$; McNemar's test).

control measures without any delay. Besides the rapid response, both SGBRT-PCR and PSRT-PCR including bacteriology were proved to be more economical per sample ($10). This price was considered "acceptable" by the poultry sector, particularly when the tests were applied in breeder flock screenings.

Throughout this study, we used both bacteriology and PCR for the detection of *Salmonella* in intestinal samples, cloacal swabs, drag swabs, litter samples, and chick dust samples, in parallel to previous detection reports from faecal (Cohen *et al*., 1994; Makino *et al*., 1999), environmental (Tuchili *et al*., 1996; Soumet *et al*., 1999; Leon-Velarde *et al*., 2004) samples by both methods, and also from cloacal swabs (Bichler *et al*., 1996; Allen-Vercoe & Woodward, 1999) by bacteriology. In our study, regardless of the method used, we obtained consistently low *Salmonella* detection rates in the first year, followed by no detection in the following years from cloacal swabs, whereas we were getting satisfactory results from other sample types, particularly from intestinal samples. We speculated that the main reason for this could be due to the low amount of faecal contamination of the cloacal swabs from infected chickens. To overcome this hindrance, we recommended the poultry companies to take proper cloacal swabs with sufficient amount of faecal contamination.

Throughout the entire study, continuous detection of *S*. Enteritidis regardless of the sample type or the year shows that this pathogen is persistently present in the poultry-related environments in Turkey. This indicates that there is still a failure in the application of general precautions or taking biosecurity actions against *S*. Enteritidis in these areas of concern.

As a conclusion, our 4-year experience revealed that the optimized PCRs with supportively applied bacteriology provided us with the opportunity to give completely satisfactory results to poultry sector to determine the *Salmonella* load in their flocks.

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Non-English Abstracts

Salmonella profile in chickens determined by real-time polymerase chain reaction and bacteriology from years 2000 to 2003 in Turkey

Aysegul Eyigor[1], Gulsen Goncagul[2], Elcin Gunaydin[3] and K. Tayfun Carli[3]*

[1]*Department of Food Hygiene and Technology,* [3]*Department of Microbiology, Faculty of Veterinary Medicine, and* [2]*Yenisehir Ibrahim Orhan Vocational High School, Uludag University, Gorukle Kampusu, 16059 Bursa, Turkey*

Entre les années 2000 et 2003, des salmonelles ont été étudiées à partir de 1785 échantillons qui comprenaient des échantillons d'intestin de poulet, des écouvillons cloacaux, des chiffonnettes de sol, des échantillons de litière et des échantillons de poussières d'élevage. Ces échantillons ont été prélevés dans 191 troupeaux de reproducteurs de volailles appartenant à 15 sociétés différentes de reproducteurs de poulet dans la région de Marmara en Turquie. Ces échantillons ont fait l'objet d'une détection par PCR en temps réel basée sur la technologie SYBR Green (SGBRT-PCR), et par une sonde spécifique (PSRT- PCR) ainsi que par une analyse bactériologique standard comme celle décrite dans le manuel du Plan National d'Amélioration des Volailles et des Dispositions Auxiliaires de l'USDA. A partir de 1242 échantillons prélevés entre janvier 2000 et juillet 2001, les taux de détection des salmonelles ont été de 5,87% et 4,10%; respectivement par SGBRT-PCR et bactériologie. A partir de 543 échantillons prélevés entre juillet 2001 et décembre 2003, les taux de détection des salmonelles ont été de 11,42 % et 5,52 %, respectivement par PRST-PCR et bactériologie. Le sérovar dominant a été *Salmonella enterica* subsp. *enterica* Serovar Enteritidis (*S*. Enteritidis), alors que les sérogroupes C1 et C2 en 2001 et le sérogroupe E1 en 2002 ont été isolés comme des sérovars additionnels. En guise de conclusion, *S*. Enteritidis semble être le problème majeur dans les troupeaux de reproducteurs en Turquie et les deux techniques de PCR en temps réel se sont révélées être plus sensibles que la bactériologie standard pour la détection des salmonelles à partir des échantillons de poulet.

In den Jahren 2000 bis 2003 wurden 1785 Proben bestehend aus Darmabstrichen, Kloaken- und Schlepptupfern von Hühnern, Einstreuproben und Hühnerstaubproben, die in 191 Hühneraufzuchtherden von 15 verschiedenen Hühnerzuchtfirmen in der Marmara-Region der Türkei gesammelt worden waren, mittels einer SYBR Green Based Real Time- Polymerasekettenrektion (SGBRT-PCR), einer Probe Specific Real Time PCR (PSRT-PCR) und mittels standardisierter, im Handbuch der National Poultry Improvement Plan and Auxiliary Provisions, USDA beschriebener bakteriologischer Methoden auf Salmonellen untersucht. Im Zeitraum zwischen Januar 2000 und Juli 2001 wurden Salmonellen mittels der SGBRT-PCR in 5,87 % und mit bakteriologischen Untersuchungen in 4,1 % von insgesamt 1242 Proben nachgewiesen. Von Juli 2001 bis Dezember 2003 wurden mit Hilfe der PSRT-PCR in 11,42 % und mit Hilfe der Bakteriologie in 5,52 % von insgesamt 543 Proben Salmonellen gefunden. Als vorherrschendes *Salmonalla*-Serovar wurde *Salmonella enterica* subsp. e*nterica* Serovar Enteritidis (S. Enteritidis) festgestellt. Außerdem wurden in 2001 die Serogruppen C1 und C2 und in 2002 die Serogruppe E1 isoliert. Zusammenfassend kann gesagt werden, dass S. Enteritidis das Hauptproblem in den Geflügelzuchtbetrieben in der Türkei zu sein scheint und dass beide angewendeten Real Time-PCR-Methoden sensitiver waren für den Nachweis von Salmonellen in Geflügelproben als die standardisierten bakteriologischen Untersuchungsmethoden.

Desde el año 2000 hasta el 2003, se investigó la presencia de *Salmonella* en un total de 1785 muestras, entre muestras intestinales de pollo, hisopos cloacales, hisopos orales, muestras de la cama y polvo de polluelos, recogidos de 191 lotes de reproductoras pertenecientes a 15 compañías diferentes de la región de Marmara, en Turquía, mediante las pruebas de reacción en cadena de la polimerasa a tiempo real SYBR Green (SGBRT-PCR), reacción en cadena de la polimerasa a tiempo real con sonda específica (PSRT-PCR) y mediante las técnicas bacteriológicas convencionales descritas en el manual del *National Poultry*

*To whom correspondence should be addressed. Tel: + 90 224 4429200, ext. 137. Fax: + 90 224 4428025. E-mail: tayfun@uludag.edu.tr
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Improvement Plan and Auxiliary Provisions, del USDA. Entre enero del 2000 y julio del 2001, se detectó *Salmonella* en un 5.87% y 4.10% de un total de 1242 muestras, por SGBRT-PCR y bacteriología, respectivamente. Desde julio del 2001 hasta diciembre del 2003, se encontró *Salmonella* en un 11.42% y 5.52% de un total de 543 muestras por PSRT-PCR y bacteriología, respectivamente. El serovar dominante de *Salmonella* fue *Salmonella enterica* subsp. e*nterica* serovar Enteritidis (S. Enteritidis), mientras que el serogrupo C1 y C2 en el 2001 y el serogrupo E1 en el 2002 fueron aislados como serovares adicionales. En conclusión, *S*. Enteritidis parece ser el mayor problema en aves reproductoras en Turquía, y ambos métodos de PCR resultaron más sensibles para la detección de *Salmonella* que las pruebas estándar de bacteriología en muestras de pollos.