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Salmonella Enteritidis predominance determined by serotyping and real-time PCR in poultry-derived food and avian isolates

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Abstract: This study aimed to determine *Salmonella enterica* subspecies *enterica* serovar Enteritidis (SE) presence by conventional serotyping and SE-specific real-time PCR (SE-rPCR) in poultry-derived food and avian isolates in our laboratory *Salmonella* spp. collection. Conventional serotyping indicated that 32 (8 chicken meat, 10 egg, 14 avian) out of 56 (57%) isolates were SE, whereas 8 (3 chicken meat, 2 turkey meat, 3 avian) (14%) isolates were serogroup B, 6 (1 chicken meat, 1 egg, 4 avian) (11%) were serogroup C1, 4 (3 chicken meat, 1 turkey meat) (7%) were serogroup C2-C3, 4 (3 chicken meat, 1 turkey meat) (7%) were serogroup E4, and 2 (avian) (4%) isolates were categorized as nonserogrouped/nonserotyped. Thirty-three (8/18 chicken meat, 10/11 egg, 15/23 avian) out of 56 (59%) *Salmonella* isolates were positive by SE-rPCR. SE was determined as the most prevalent serotype in both of the tests regardless of the sample type. Conventional serotyping and SE-rPCR results were in agreement in all but 1 isolate. Considerably high relative accuracy (98%), sensitivity (100%), and specificity (96%) with almost perfect agreement between the two methods (Cohen's kappa = 0.96) indicated SE-rPCR as a reliable tool in the rapid identification of SE isolates to complement conventional serotyping.

Key words: *Salmonella* Enteritidis, real-time PCR, serotyping, poultry meat, egg, avian

1. Introduction

Human salmonellosis is one of the most frequently encountered food-borne bacterial diseases worldwide. Two nontyphoidal *Salmonella* (NTS) serovars, *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis, SE) and *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium, ST), account for the majority of the cases, which range from asymptomatic colonization to severe systemic infections. Major sources of these two important NTS serovars are reported as subclinically infected or intermittent/persistent carrier farm animals and their products, where human SE infections are primarily related to the consumption of contaminated poultry meat and eggs (1). Therefore, national control and surveillance programs in the EU (2) and in Turkey (3) have been implemented in order to control *Salmonella* serovars including SE in these high-risk sources.

Worldwide, *Salmonella* serotyping is performed by the White–Kauffmann–Le Minor classification scheme as the gold standard, allowing long-term epidemiological surveillance of this pathogen in the food chain and in public health control. This useful but time-consuming and labor-intensive method, despite its inherent disadvantages, is commonly used in initial screening of the isolate, followed by molecular subtyping for strain identification (4,5). Therefore, for rapid detection of SE and other frequently reported *Salmonella* serotypes, alternative serotype-specific conventional or real-time PCRs (rPCRs) were performed and reported (6–9) to augment taking immediate actions related to public health.

This study aims to determine SE presence by conventional serotyping and SE-specific real-time PCR (SE-rPCR) in poultry-derived food and avian isolates in our laboratory *Salmonella* spp. collection. In conventional serotyping, we selected a limited number of commercially available antisera in an effort to primarily identify the serogroup of the isolate, and then performed serotyping based on current serotype distribution data reporting SE as one of the most commonly isolated serotypes in Turkey (10–14).

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2. Materials and methods

2.1. Standard strains

S. Enteritidis 64K (MY Popoff, Institut Pasteur, Paris, France), *S.* Typhimurium NCTC 12416 (Refik Saydam National Public Health Agency, Ankara, Turkey), and six non-*Salmonella* isolates (*Citrobacter* sp., *Escherichia coli*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Streptococcus* sp., *Proteus* sp.) from the Department of Microbiology, Medical School, and Faculty of Veterinary Medicine, Uludağ University, Bursa, Turkey, were used as positive and negative controls in SE-rPCR and serogrouping/typing, respectively.

In this study, 56 *Salmonella* isolates, comprising 33 poultry-derived food (18 chicken meat- wing, whole chicken, deboned leg with skin, drumstick; 4 turkey meat-neck; 11 egg- inner and shell) and 23 avian (9 chicken feces, 3 chicken cloacal swab, 1 crow intestine, 3 chick intestine, 1 chicken eye, 6 chicken drag swab), which were isolated, collected in different studies between 2000 and 2015, and stored in the Uludağ University Faculty of Veterinary Medicine Microbiology and Food Hygiene and Technology Department Laboratories, were used.

2.2. Serogrouping/serotyping

Serotyping was performed on the basis of reaction with O- and H-group antigen, according to the White–Kauffmann–Le Minor classification scheme (4) and Guibourdenche et al. (5) by using commercial antisera (Becton Dickinson- BD and Statens Serum Institute- SSI) available in our laboratory. For conventional *Salmonella* identification, particular O antisera (Salmonella O Poly Antisera; Salmonella O Grouping Antisera, BD) and H antisera (Salmonella H Spicer Edwards Antisera, BD; Salmonella H Antisera, BD and SSI) were used. Briefly, for serogrouping, each isolate grown on nutrient agar (NA) (Oxoid, CM0003) was tested by slide agglutination with Polyvalent *Salmonella* O Antisera, and further with Monovalent *Salmonella* O Group Antisera. After serogroup identification, before serotyping, several consecutive transfers of each isolate were performed in Motility GI Medium (BD, 286910) in order to increase its motility. For serotyping, after motility determination, each isolate grown on brain heart infusion broth (Oxoid, CM1135) was tested with *Salmonella* H Spicer Edwards antisera (including a combination of polyvalent and single complex antisera) by tube agglutination (BD) to screen and identify the most commonly encountered *Salmonella*, and then by related *Salmonella* H antisera using either tube agglutination (BD) or slide agglutination (SSI) according to the manufacturer's instructions. Quality control antigens QC Antigen *Salmonella* O group and *Salmonella* Vi (BD) were used as positive controls in serotyping.

2.3. Template preparation for SE-rPCR

Crude template DNA was extracted from each control strain and isolate grown on NA (Oxoid, CM0309) according to the procedure described by Carli et al. (15). Briefly, a loopful of each pure culture was suspended in 100 µL of 0.85% NaCl and washed twice, and the final pellet was resuspended in 20 µL of deionized water. This suspension was then boiled for 10 min and centrifuged for 3 min at 18,000 × *g*, and 2 µL of the supernatant was used as a template in rPCR. Concentrations and purity determinations of the template DNA were performed with a NanoDrop spectrophotometer (ND1000, Thermo Scientific, Waltham, MA, USA).

Nucleotide sequences of the forward primer SefB596614 and TaqMan probe SEFB_640660 (both selected in this study), and the reverse primer SefB661R (previously described by Wang et al., 2009), which were based on the *S.* Enteritidis fimbria (*sef*b) gene (accession number L11009), were as follows, respectively: 5' - ATA TTA AAT CTG GTA ATT T - 3'; 5' - FAM - GCATATCCAAATGGCTCAAT-TAMRA - 3'; 5' - TGT ACT CCA CCA GGT AAT TG - 3'. The internal amplification control (IAC) sequence (CGT CAG TGT GAA GCG GTT ATA AAT CTG CTC TTT CGC GGT ATC CGT ACC GAT TTC GGT AAG GTA AAC CCC GTT TTT GTT TCG CTT ACG TGG CAT) in SE-rPCR was designed based on a sequence specific to the lambda phage of *E. coli* and had a specific probe and primers as follows: IAC probe sequence 5' - HEX - TGC TCT TTC GCG GTA TCC GTA CCG AT - TAMRA - 3'; forward primer 5'- CGT CAG TGT GAA GCG GTT ATA A - 3'; reverse primer 5'- ATG CCA CGT AAG CGA AAC A -3' (Way2Gene, BN 15-0001-01, Genmar, Turkey). All primers and probes were included in a custom-made system according to our specifications (utilizing predefined sequences and our bacterial DNAs as positive and negative controls) with a determined specificity and detection limit of 100% and 3 CFU mL^{-1}, respectively, by the manufacturer (SE-rPCR kit, Salmonella 5' nuclease/TaqMan rPCR, Way2Gene, BN 15-0001-01, Genmar, Turkey). Ten microliters of reaction mix for SE-rPCR performed in a LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany) comprised 2.5 µL of detection mix (parameter-specific reaction mix including primers and probes), 1 µL of IAC DNA, 2 µL of PCR-grade water, 2 µL of enzyme mix (enzyme, dNTP mix, reaction buffer), and 2.5 µL of template DNA (or 2 µL of ultrapure water for no-target control). The amplification protocol was initiated with a denaturation step at 95 °C for 11 min, followed by 40 cycles of 10 s of denaturation at 95 °C, 30 s of annealing at 58 °C, and 5 s of primer extension at 72 °C. The temperature transition rate was 20 °C/s. Fluorescence values of each sample and IAC were automatically measured at 530 nm and at 560 nm at the end of each

annealing step. Data analysis was automatically performed by the LightCycler software version 4.05 (Roche).

2.4. Statistical analysis

Relative accuracy, sensitivity, and specificity were calculated according to the protocol described in ISO 16140:2003 (16). Relative accuracy, sensitivity, and specificity calculations were complemented with Cohen's kappa test to evaluate the correspondence between results obtained by methods.

3. Results

Conventional serotyping indicated that 32 out of 56 (57%) isolates were SE, whereas 8 (14%) isolates were serogroup B, 6 (11%) were serogroup C1, 4 (7%) were serogroup C2-C3, 4 (7%) were serogroup E4, and 2 (4%) isolates were categorized as nonserogrouped/nonserotyped (NS). When results were analyzed based on sample type, there were 33 poultry-derived food isolates, from which 18 (55%) were serotyped as SE and the remaining 5 isolates were in serogroup B (15%), 2 isolates were in serogroup C1 (6%), 4 isolates were in serogroup C2-C3 (12%), and 4 isolates were in serogroup E4 (12%). In a total of 23 avian isolates, 14 (60.9%), 3 (13%), 4 (17.4%), and 2 (8.7%) were serotype SE, serogroup B, serogroup C1, and NS, respectively. Thirty-three out of 56 total *Salmonella* isolates (59%) were positive by SE-rPCR. When results were evaluated based on sample type, 18 out of 33 (55%) poultry-derived food isolates and 15 out of 23 (65.2%) avian isolates were positive in SE-rPCR, while 15 (45%) and 8 (34.8%) isolates were negative, respectively. Conventional serotyping and SE-rPCR results were in agreement in all but 1 of the isolates tested. One crow intestine isolate (no. 250), which was identified as SE by SE-rPCR, was classified under NS by the available antisera in this study (Table).

Following the ISO 16140 statistical protocol (16), when SE-rPCR was compared to conventional serotyping as the reference method, there was only 1 false positive and no false negative result in SE-rPCR with respect to conventional serotyping. Therefore, the relative accuracy, sensitivity, and specificity of SE-rPCR were considerably high with respect to the reference method at 98%, 100%, and 96%, with almost perfect agreement between the two methods (Cohen's kappa = 0.96).

4. Discussion

In this study, serotyping results revealed SE predominance in a total of 56 (57%) isolates in our laboratory *Salmonella* spp. collection of poultry-derived food and avian origin. More than half (55%) of the poultry-derived food isolates were determined as SE, and, when examined in detail, serotyping of 18 chicken meat isolates indicated that 8 were SE, while 3 isolates per each serogroup were in B, C2-C3, and E4, and 1 isolate was in serogroup C1 (Table). This finding denoting SE as the most prominent serotype in chicken meat isolates is similar to the findings of others (10,13,17–19). There are studies reporting the SE prevalence in chicken carcasses and retail whole chicken or meat parts similar to (10), close to but higher than (17,19), or lower than (13,18) our SE result of 44.4%. In contrast to our high SE (serogroup D) prevalence rate in chicken meat isolates, there are studies where the most common serogroups were reported as serogroup B (20), serogroup C1 (21), serogroup C2-C3 (22), and serogroup E (E4) (13). As seen from the Table, none of the 4 turkey meat isolates tested were SE, and they were serogrouped in B, C2-C3, and E4. There are similar studies reporting other serogroup prevalences with no SE isolation (13,23–25) from turkey meats. High serogroup B detection rates were reported by Folster et al. (24) and Kinross et al. (25), who also implied an increase in the isolation of serovars within serogroup B in their samples. There are other studies with the highest prevalence of serogroup C2-C3 in turkey meat samples (23). The presence of *Salmonella* serogroups other than serogroup D in turkey meats compared to a higher SE presence in chicken meat samples in this study may once more indicate chicken meat as a major SE reservoir for human infections. Serotyping 10 out of 11 *Salmonella* egg isolates as SE (91%) and only 1 isolate as C1, regardless of the egg part sampled, is good evidence supporting the SE predominance in eggs as indicated previously (14,26). This serovar is widely studied since it represents the dominant serotype involved in foodborne diseases due to eggs or egg product consumption (1). However, in a recent study by Lublin et al. (27), a high prevalence of serogroup C1 in retail table eggs opposed to no detection of SE was reported. Regardless, our results indicate that SE continues to be the most important serovar in Turkey both for layers and in retail eggs.

There was a SE dominance with a rate of 60% in avian isolates, similar to the poultry-derived food isolates. Apart from the high detection rate of SE in chicken feces and cloacal swab isolates, some isolates were found to be in serogroups B and C1, whereas only 2 isolates (crow and chick intestine) were NS with the available antisera (Table). In previous studies, which used similar avian sample types such as cloacal swabs, feces, and intestine, detection of SE was previously indicated by Kinde et al. (28), while a higher prevalence of other serogroups such as B (29) and C1 (26) was also reported. As another serotyping finding in our study, all chicken drag swab isolates were SE, which, although contrasted to the relatively lower SE incidences of Kinde et al. (28) and Kahya et al. (11), is in agreement with other previous reports that suggested the drag swab as an effective sample type in SE detection from environmental samples of layer flocks (28,29). The prevalence and serogrouping/typing differences observed in our poultry-

Table. SE-rPCR and serological results of *Salmonella* isolates of poultry-derived food and avian origin.

Sample type	No. of samples (sample ID)	SE-rPCR		Serogroup					NS
Poultry-derived food		Negative	Positive	B (O:4)	C1 (O:7)	C2-C3 (O:8)	D1 (O:9)	E4 (O:1,3,19)	
Chicken meat									
Wing	5 (5, 6, 8, 12, 99)	1 (99)	4 (5, 6, 8, 12)		1 (99)		4 (**5**, **6**, **8**, **12**)		
Whole chicken	3 (20, 21, 28)	1 (21)	2 (20, 28)			1 (21)	2 (**20**, **28**)		
Deboned leg with skin	6 (47, 54, 94, 95, 96, 97)	4 (94, 95, 96, 97)	2 (47, 54)	1 (94)		2 (95, 96)	2 (**47**, **54**)	1 (97)	
Drumstick	4 (90, 92, 98, 100)	4 (90, 92, 98, 100)		2 (90, 92)				2 (98, 100)	
Total chicken meat	18	10	8	3	1	3	8	3	
Turkey meat									
Neck	4 (75, 76, 78, 88)	4 (75, 76, 78, 88)		2 (75, 76)		1 (78)		1 (88)	
Total turkey meat	4	4		2		1		1	
Egg									
Inner	8 (122, 131, 148, 151, 152, 153, 154, 155)	1 (131)	7 (122, 148, 151, 152, 153, 154, 155)		1 (131)		7 (**122**, **148**, **151**, **152**, **153**, **154**, **155**)		
Shell	3 (139, 146, 147)		3 (139, 146, 147)				3 (**139**, **146**, **147**)		
Egg total	11	1	10		1		10		
Poultry-derived food total	33	15	18	5	2	4	18	4	
Avian									
Chicken feces	9 (173, 271, 273, 275, 287, 288, 289, 292, 298)	5 (173, 271, 273, 287, 288)	4 (275, 289, 292, 298)	3 (271, 273, 287)	2 (173, 288)		4 (**275**, **289**, **292**, **298**)		
Chicken cloacal swab	3 (202, 220, 249)	2 (202, 220)	1 (249)		2 (202, 220)		1 (**249**)		
Crow intestine	1 (250)		1 (250)						1 (250)
Chick intestine	3 (251, 253, 259)	1 (259)	2 (251, 253)				2 (**251**, **253**)		1 (259)
Chicken eye	1 (254)		1 (254)				1 (**254**)		
Chicken drag swab	6 (BA, B98, B99, B100, B104, B128)		6 (BA, B98, B99, B100, B104, B128)				6 (**BA**, **B98**, **B99**, **B100**, **B104**, **B128**)		
Avian total	23	8	15	3	4		14		2
Total (%)	56	23 (41)	33 (59)	8 (14)	6 (11)	4 (7)	32 (57)	4 (7)	2 (4)

NS: Could not be serogrouped/serotyped with available antisera.

Bolded and underlined isolates in parentheses: serotyped as *Salmonella enterica* subspecies *enterica* serovar Enteritidis/

derived food and avian isolates in this study could be mainly related to the production system used; differences in slaughtering and cooling processes; variations in market types and storage conditions; differences in sampling period, year, and region; sampling methods; sample type; and isolation and identification method used (13,20,26).

SE-rPCR results of the 56 total *Salmonella* isolates indicated that 33 (59%) and 23 (41%) of the isolates were positive and negative, respectively. This finding in rPCR is parallel to the serotyping finding of SE dominance of 57%. When results were evaluated based on sample type, 18 out of 33 (55%) poultry-derived food isolates that were serotyped as SE were also positive in SE-rPCR. As shown in the Table, the remaining 15 (45%) SE-rPCR negative isolates were not identified as SE and were in other serogroups. A recent study from Turkey by Dumen et al. (12) also reported SE as the most prevalent (26.6%) serotype both by PCR and by conventional serotyping. In contrast, this serovar was either not detected (7,8) or was detected in very low rates (6) from chicken meat-related or egg samples by conventional or real-time PCR in different studies.

Out of 23 avian isolates, 15 (65.2%) were positive and 8 (34.8%) were negative in SE-rPCR. Serogrouping results of the same isolates indicated that 14 (except one false positive result, isolate no. 250) of these isolates were SE, while 9 (one additional isolate NS in serogrouping, no. 259) were in other serogroups (Table). Since isolate no. 259, which was negative in SE-rPCR, was not serotyped as SE (NS in serogrouping), it was not regarded as a false negative in the statistical evaluation. Our results are similar to those of Lungu et al. (9), who found 67.1% and 61.3% of the boot sock and drag swab samples as SE-positive by real-time PCR.

When conventional serotyping and SE-rPCR results were examined, from a total of 56 *Salmonella* isolates, 32 (57%) and 33 (59%) were determined as SE by serotyping and SE-rPCR, respectively. The disagreement between these findings comes from 1 isolate (no. 250, crow intestine), which was NS in serogrouping but detected as SE in rPCR (Table). Since conventional serotyping is considered as the reference method for determining the serovar of the isolate, positive detection in SE-rPCR was regarded as a false positive for this test. One possible reason for this false positivity in PCR could be related to the loss of antigen expression (e.g., due to repeated subculturing) leading to strain untypability in serotyping (30). Other underlying factors for this false positivity coinciding particularly to this NS isolate in SE-rPCR, and to which serogroup/serotype it belonged, was not in the scope of this study and requires further investigation. Differences in the detection rate of SE by rPCR in this study compared to the findings of others could mainly be related to the country and prevalence of the serovar in that region during that time period. In addition, sample size and type, and the validity of the PCR detection system (specificity, sensitivity, accuracy/reliability), can be the main factors among others affecting this outcome (7,8,30).

The relative accuracy, sensitivity, and specificity of SE-rPCR was considerably high with respect to conventional serotyping, and the agreement between the methods was almost perfect as indicated by a high Cohen's kappa index ($\kappa = 0.96$). This could be mainly related to using pure culture isolates, and to the compatibility and specificity of the primers used in our PCR system, which was designed for the detection of SE. There can be many other interrelated factors in designing PCR systems (gene-specific primer selection, use of internal control, prevention of PCR inhibition) for the detection of different/multiple serovars from different matrices with/without enrichment cultures, which challenge the relative accuracy of the tests as indicated by others (7–9).

In conclusion, serotyping 32 (57%) of the 56 *Salmonella* isolates as SE indicated the predominance of this serotype in poultry-derived food and avian isolates. SE-rPCR, which detected 33 (59%) SE isolates, can be considered as a reliable tool in primary and rapid screening for SE in endemic areas and plants, complemented by the gold standard of conventional serotyping in the identification of *Salmonella* in order to confirm rare false PCR results.

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