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Research Article

Turk. J. Vet. Anim. Sci.
2010; 34(2): 181-186
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doi:10.3906/vet-0810-43

Identification and characterization of *Salmonella* isolates from captured house sparrows

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Received: 30.10.2008

Abstract: The objectives of this study were to determine the prevalence of *Salmonella* in a population of house sparrows, which are commonly found around poultry houses, and to characterize the obtained *Salmonella* isolates via serotyping, multiplex polymerase chain reaction (PCR), and antibiotic resistance analysis. Samples of visceral organs (gastrointestinal tract, liver, and heart) from 470 house sparrows were subjected to culture and the results show that 18 (3.8%) were positive for *Salmonella*. Of the 18 *Salmonella* isolates characterized, the most predominant serovars were *Salmonella* Typhimurium and *S.* Enteritidis (9 and 8 cases each, respectively), whereas only 1 serovar belonged to *S.* Montevideo. All 9 *S.* Typhimurium serovars were positive for *rfb*J, *flj*B, *inv*A, and *fli*C genes based on multiplex PCR assay. In the case of *S.* Enteritidis serovars, PCR generated amplification products for *spv* and *sef*A genes, and a random sequence (specific for the genus *Salmonella*) in all 8 samples. All the *Salmonella* isolates were sensitive to norfloxacin, flumequine, ampicillin, and sultrim, and 35% were resistant to lincospectin (the most prevalent resistance).

Key words: *Salmonella,* sparrows, serotyping, multiplex PCR

Introduction

Salmonella are zoonotic pathogens, as they can cause food-borne illness in humans. The species *Salmonella enterica* comprises 6 subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*, otherwise known as subspecies I, II, IIIa, IIIb, IV, and VI, respectively. *S. enterica* subsp. I is usually isolated from humans and warm-blooded animals, and the majority of serotypes isolated in clinical laboratories belong to this subspecies (1). Raw meat and poultry are recognized as the primary sources of *Salmonella* species transmitted to humans, with 40% of the clinical cases attributed to the consumption of eggs and poultry products. Although *Salmonella* are generally inactivated during standard cooking practices, improper handling and preparation of meat and meat products continue to contribute to human salmonellosis cases (2). An important route for introducing *Salmonella* into poultry houses is free-flying wild birds. *Salmonella* spp., especially *Salmonella enterica* subspecies Typhimurium, are commonly found in the intestines of wild birds. These

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infected birds may transmit infection to humans either indirectly by infecting pets and food animals, or directly as a result of handling, as reported by Tizard (3). Passerines, like house sparrows, appear to be more at risk for *Salmonella* infection, as they usually live in clusters in which contamination of seeds by infected feces may occur (4,5). Small free-flying wild birds, such as wild doves, starlings, and in particular house sparrows, which live nearly anywhere but usually gather in relatively large numbers around poultry houses, have the opportunity to enter the houses or feed stores, especially if the house buildings are not properly bird-proofed; therefore, contamination of feed or water supplies and growing chickens may occur.

Although *Salmonella* spp. have frequently been found in wild birds and reports of isolation of *Salmonella* from these birds have been published worldwide, there are no reports on the identification and molecular characterization of *Salmonella* in wild birds in Iran. Identification of *Salmonella* can be performed via both serotyping and molecular methods. Serotyping offers a reliable method for differentiating *Salmonella* strains, but this procedure usually depends on using a complete set of antisera and thus can be time-consuming. In contrast, molecular methods are fast, as well as highly sensitive and very specific. In addition, molecular identification of pathogens can be used for epidemiological research. Because of the high sensitivity and specificity of molecular methods, we decided to use multiplex polymerase chain reaction (PCR) in support of the identification of *Salmonella* based on serotyping.

The main objectives of the present study were to determine the prevalence of *Salmonella* in a population of house sparrows collected near poultry farms and to characterize the isolates via serotyping, multiplex PCR, and antibiotic resistance analysis. The results obtained with traditional serotyping and molecular typing were then compared.

Materials and methods

Sample collection

During April-August 2006 we captured 470 house sparrows near industrial poultry houses in the vicinity of Tehran, the capital city of Iran. Trapped birds were euthanatized and put into plastic bags, cooled in an icebox, and immediately transported to the laboratory. Under sterile conditions in the laboratory the gastrointestinal tract (proventricle to cloaca), liver, and heart were removed and minced separately using sterile scissors. Homogenized samples of a liver-heart mixture and gastrointestinal tissues were examined for the presence of *Salmonella*.

Isolation and identification of *Salmonella*

The procedures used in the present study for the isolation of *Salmonella* were previously described (6). Homogenized samples of the liver-heart mixture were cultured in peptone water at 37 °C for 24 h. Samples of both homogenized gastrointestinal tissues and the liver-heart mixture were cultured in selenite F medium. These samples were incubated at 37 °C for 18 h, and then each sample was inoculated onto Salmonella-Shigella agar (SS) and Brilliant Green agar (BG) plates. The plates were incubated at 37 °C for 24 h. Suspicious colonies morphologically similar to *Salmonella* were subcultured for biochemical examination. Identification of the biochemical characteristics was performed using triple sugar iron (TSI) medium, urea medium, lysine-iron agar (LIA) medium, Simmon's citrate medium, motility medium, and lactose, saccharose, maltose, and mannitol media.

Serotyping

Eighteen *Salmonella* isolates obtained from the visceral organs of house sparrows were used for serotyping. The *Salmonella* isolates were first cultured onto TSI slant medium and grown overnight at 37 °C, and then were tested using antisera O (B, D, E, C) and H based on slide and tube agglutination tests to determine O and H antigens, respectively (6).

Multiplex PCR

Nine *S*. Typhimurium strains, 8 *S*. Enteritidis strains, and 1 *S*. Montevideo strain isolated from house sparrows were cultured onto Luria Bertani (LB) agar plates and incubated at 37 °C for 24 h. For DNA extraction, 1 loopful of each sample from LB agar was suspended in 250 µL of sterile distilled water. In order to have uniform turbidity the samples were vortexed, and then were boiled for 10 min and centrifuged at 6000 ×g for 7 min. Supernatants were collected and saved for multiplex PCR analysis.

Multiplex PCR was performed with 2 independent sets for DNA amplification of *S.* Typhimurium and *S.* Enteritidis. As a comparison, only 1 *S.* Montevideo was included in the PCR set for *S.* Enteritidis. Four sets of primer pairs specific for *rfb*J (663 bp), *flj*B (526 bp), *inv*A (284 bp), and *fli*C (183 bp) were used in the case of *S.* Typhimurium (Table 1), and 3 sets of primer pairs designed for a random sequence specific for the genus *Salmonella* (429 bp), *sef*A (310 bp), and *spv* (250 bp) for *S.* Enteritidis (7) were used (Table 2). Both reactions were performed in a final volume of 25 μL that contained 4 μL of template DNA, 2.5 μL of reaction buffer (10´), 0.8 μL of dNTPs (10 mM), 1 μL of $MgCl_2$ (50 mM), 0.3 μL of Taq polymerase (5 U μL^{-1}), 8.4 μL of sterile distilled water, and 1 μL of each primer (10 μM) for *S.* Typhimurium, and for *S.* Typhimurium 3 μL of template DNA, 0.6 μL of Taq polymerase, 9.9 μL of distilled water, and 1.25 μL of each primer (10 μM), in addition to buffer, dNTPs, and $MgCl_2$ at the same volume and stock concentrations as mentioned.

Amplification was carried out using a Techne TC-512 thermocycler (Techne, UK), as follows: 35 cycles of 30 s for denaturation at 94 °C, 90 s for annealing at 56 °C, and 30 s for primer extension at 72 °C, followed by a terminal extension at 72 °C for 10 min in the case of *S.* Enteritidis. Target genes for *S.* Typhimurium were amplified using the same thermocycler, as follows: 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, primer extension at 72 °C for 30 s, followed by 7 min at 72 °C for terminal extension. For both amplifications, initial denaturation at 95 °C for 5 min was used.

The amplification products were analyzed by agar gel electrophoresis. Electrophoresis of the amplification products was performed on 1.2% and 1.8% agarose gel for *S.* Typhimurium and *S.*

Table 1. Primers used for the detection of *Salmonella* Typhimurium.

Primer	Target gene	Length	Sequence (5´-3´)	Amplification product (bp)
ST139-s	*inv*A	26	GTGAAATTATCGCCACGTTCGGGCAA	284
ST141-as	*inv*A	22	TCATCGCACCGTCAAAGGAACC	
Rfbj-s	*rfb*J	24	CCAGCACCAGTTCCAACTTGATAC	663
Rfbj-as	*rfb*J	24	GGCTTCCGGCTTTATTGGTAAGCA	
Flic-s	*fli*C	23	ATAGCCATCTTACCAGTTCCCCC	183
Flic-as	*fli*C	24	GCTGCAACTGTTACAGGATATGCC	
Fljb-s	*flj*B	24	ACGAATGGTACGGCTTCTGTAACC	526
Flbj-as	*flj*B	24	TACCGTCGATAGTAACGACTTCGG	

Table 2. Primers used for the detection of *Salmonella* Enteritidis.

Primer	Target gene	Length	Sequence (5´-3´)	Amplification product (bp)
ST11	Random sequence*	24	GCCAACCATTGCTAAATTGGCGCA	429
ST14	Random sequence	25	GGTAGAAATTCCCAGCGGGTACTGG	
S1	*Spv***	20	GCCGTACACGAGCTTATAGA	250
S4	*spv*	20	ACCTACAGGGGCACAATAAC	
SEFA2	*sef*A****	20	GCAGCGGTTACTATTGCAGC	310
SEFA4	*sef*A	20	TGTGACAGGGACATTTAGCG	

* = Randomly cloned sequence specific for the genus *Salmonella*
** = *Salmonella* plasmid virulent gene
*** = *S.* Enteritidis fimbrial antigen gene

Enteritidis samples, respectively. In both reactions, a 100-bp ladder was used as a molecular weight marker. The gels were stained with ethidium bromide (2 μg mL^{-1}) to visualize fluorescent bands while using UV in the gel document system (BIORAD, UK).

Antibiotic resistance analysis

The antimicrobial susceptibility of the 18 *Salmonella* isolates to 8 antibiotics was determined using the disk diffusion method, as described by the National Committee for Clinical Laboratory Standards (Table 3) (8). Each *Salmonella* isolate was cultured on tryptose soy broth (TSB) and incubated at 37 °C for a few hours, and then was calibrated based on the 0.5 McFarland $BaSO_4$ turbidity standard. Each adjusted turbidity sample was then transferred to Mueller Hinton agar. Antimicrobial disks were disposed on the surface of inoculated agar media aseptically and incubated at 37 °C for 18-20 h. The growth inhibition zones of each disk were measured and the results were interpreted based on comparison to standards.

Table 3. Antibiotics and their concentration in the disks.

Antibiotic name with abbreviation	Drug concentration (μg)
Florfenicol (FF)	30
Flumequine (FM)	30
Ampicillin (Amp)	10
Lincospectin (LP)	15/200
Tetracycline (TE)	30
Neomycin (N)	30
Norfloxacin (NFX)	10
Sultrim (SXT)	25

Results

Salmonella was isolated from 18 (3.8%) of the 470 samples. From these isolates, 3 serovars were identified, which included 9 *S.* Typhimurium serovars (50%), 8 *S.* Enteritidis serovars (44.4%), and 1 *S.* Montevideo serovar (5.5%). PCR produced 663, 526, 284, and 183 base pair amplification products from *rfb*J, *flj*B, *inv*A, and *fli*C, respectively, in all 9 *S.* Typhimurium serovars (Figure A). In the case of *S.* Enteritidis, PCR amplified 429, 310, and 250 base pair products from a random sequence (specific for the genus *Salmonella*), *sef*A, and *spv* in all of 8 samples (Figure B). The only serologically identified serotype (*S.* Montevideo) was positive for the random sequence specific for the genus *Salmonella*, but was negative for both *sef*A- and *spv*-specific bands.

The results of antibiotic resistance analysis show that all the *Salmonella* isolates were sensitive to norfloxacin, flumequine, ampicillin, and sultrim. Resistance to neomycin and florfenicol was not observed, although some isolates had intermediate sensitivity to these antimicrobials (Table 4). Among the 3 identified serotypes, 6 Typhimurium isolates and 1 Montevideo isolate were antibiotic resistant. When

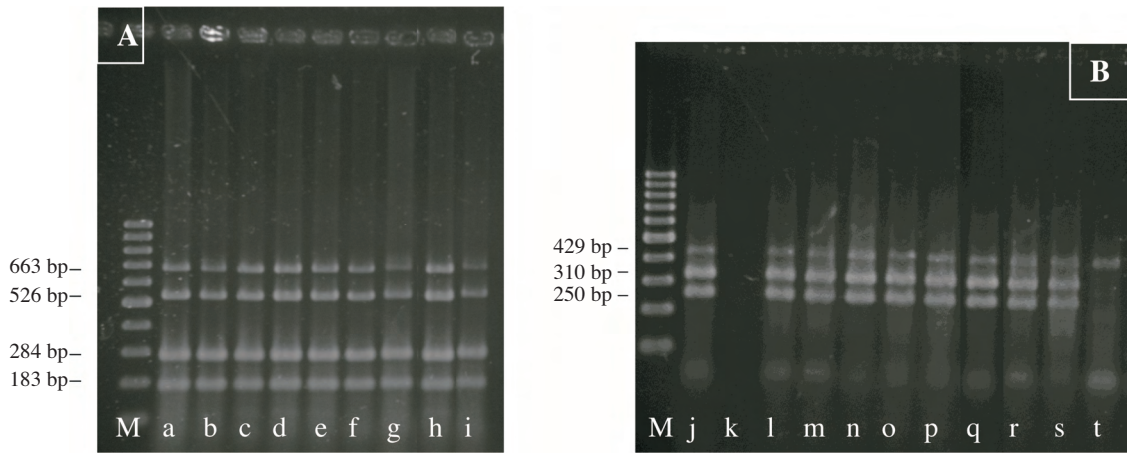

Figure. Multiplex polymerase chain reaction for detection of S. Typhimurium (A) and S. Enteritidis (B). Lane M: 100 bp ladder. Lane a-i: ST isolates. Lane j: positive standard for S. Enteritidis. Lane k: blank. Lanes l-s: SE isolates. Lane t: S. Montevideo.

Table 4. Serotypes of *Salmonella* isolates and the antibiotic resistance profile.

Serotype	Isolates (n)	Percentage of isolates (%)	Resistant isolates (n)	Resistance profile*
Typhimurium	9	50	6	LP
Enteritidis	8	44.5	0	NR**
Montevideo	1	5.5	1	LP/TE
Total	18	100	7	

* Antibiotics are defined in Table 3
** No resistance

expressed as a percentage of resistance, 66.6% (6 of 9) and 100% (1 of 1) of the Typhimurium and Montevideo samples exhibited antibiotic resistance, respectively.

Discussion

Salmonella isolates obtained from house sparrows and other wild birds have been reported from all over the world (9-11). In a study conducted in the United States in July and August 1997, 25 wild bird intestinal and fecal samples were collected from a broiler house, and 6 samples (24%) were positive for *Salmonella* spp. (12). Between 1984 and 1991 the incidence of *Salmonella* was studied in wild birds from various sites in the Czech Republic, including agricultural farms and natural habitats. *Salmonella* was isolated from 25% of the examined birds (including house sparrows), and from 4.2% and 19.2% of the examined adult and young black-headed gulls, respectively (13). Results of another study carried out with the carcasses of 779 wild birds (including house sparrows) in Great Britain between 1995 and 2003 showed that *Salmonella* Typhimurium was the most predominant serovar in the wild birds (14). Kapperud et al. (15) suggested that serovar Typhimurium has established a reservoir in avian wildlife in Norway, and epidemiological and bacteriological evidence indicate that wild birds may transmit the infection to humans and to poultry. These reports indicate that strains of *Salmonella* spp. in wild birds could correlate with strains isolated from domestic animals and chickens.

According to Rezania (unpublished data), the prevalence of *Salmonella* was 6% (18 of 300) in house sparrows collected around poultry houses in Tehran. In that study the prevalence rate of 4 serovars, including *Salmonella* Typhimurium, *Salmonella* Gallinarum, *Salmonella* Thompson, and *Salmonella* Suberu, were reported. In comparison to Rezania's results, those of the present study show that there was a 2.2% decrease in the prevalence of *Salmonella* in the population of house sparrows in Tehran.

In the present study *Salmonella* Typhimurium was the most prevalent serovar identified (50%). According to the Centers for Disease Control and Prevention (CDC), *Salmonella* Typhimurium and Enteritidis are the 2 most common serovars associated with human disease, and are therefore of importance to public health. *Salmonella* Montevideo was also listed among the top 20 serovars identified in the CDC report (16).

Several molecular methods based on the amplification of DNA have been developed for the detection of *Salmonella* serotypes. *Salmonella* organisms are characterized by somatic (O) and flagellar (H) antigens. The genes for O-antigenic synthesis are normally grouped together on the chromosome in the *rfb* gene cluster. The flagellar antigens H1 and H2 for diphasic serotypes, such as *S.* Typhimurium, are encoded by the *fli*C and *flj*B genes, respectively (1). The *inv*A gene is associated with *Salmonella* virulence. These 4 genes, which are used to confirm the presence of *S.* Typhimurium, were observed in all of the *S.* Typhimurium serotypes in the present study. *Spv* and *sef*A genes were also important because they could distinguish *S.* Enteritidis from *Salmonella* non-*enteritidis* strains.

The present study's results show that the correlation between traditional serotyping and multiplex PCR was 100%; thus, this molecular method can confirm the results of traditional serotyping. However, the development of a molecular

method does not necessarily imply that we should rule out traditional serotyping, because these 2 methods are complementary and the molecular method simply augments the available tools for successfully typing strains that cause health problems. The specificity and sensitivity of multiplex PCR make it a potentially valuable tool for the detection of *Salmonella* species.

Antibiotic-resistant *Salmonella* isolates are also important for public health. Of the *Salmonella* isolates we identified, 38.8% (7 of 18) were resistant to at least 1 antibiotic (Table 4). Widespread use of antibiotics to treat diseases and to promote growth by the livestock and poultry industries have resulted in the emergence of resistant strains, which has raised some concerns about the efficacy of human antimicrobial therapy, because resistant *Salmonella* strains can be introduced into the human food chain and produce resistant infections. The findings of the present study, as well as those of previous studies, show that house sparrows can harbor multiple *Salmonella* serovars with variable antibiotic resistance patterns. Thus, these birds, especially those that gain entry to poultry houses, have the potential to transmit this pathogen to poultry.

Acknowledgment

This study was supported by the Faculty of Veterinary Medicine, University of Tehran. We also gratefully acknowledge the guidance offered by A. Yazdani.

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