Characterization of *Salmonella* isolates from poultry sources in Iran

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Abstract:

BACKGROUND: Salmonellosis is one of the most important zoonotic diseases throughout the world. **OBJECTIVES:** The purpose of this study was to characterize a large collection of *Salmonella* isolates from different poultry sources in Iran. **METHODS:** A total of 123 *Salmonella* isolates from different poultry sources were subjected to drug susceptibility test, hemolysin production, motility test, and plasmid profile (50 isolates). **RESULTS:** Seventy-one resistance patterns were found to 29 antimicrobial agents among 123 *Salmonella* isolates, in which 81% of isolates were resistant to more than one antibacterial agent. The resistance patterns of 123 isolates to 10 commonly used antibacterials in Iranian poultry industry were also quite variable and included 31 patterns. Four different plasmid patterns were found among 50 *Salmonella* isolates. Fifty four percent of *Salmonella* isolates harbored one or three plasmids with approximate molecular size ranging from 2.3 to 68 kb. No plasmid was detected in 46% of isolates. A band of 68 kb size was detected in all isolates that harbored plasmid. All isolates were motile but no isolate showed hemolysin production. **CONCLUSIONS:** The frequency of resistance to antibacterial agents among avian *Salmonella* isolates is a major public health concern.

Introduction

Salmonellosis is one of the most important food-borne diseases throughout the world (Hendriksen, 2003; Valkenburgh et al., 2007). More than 2600 serovars of *Salmonella* have been identified, some of which are responsible for human illness and diseases in a wide variety of animals (Gast, 2008). Humans most often become infected after consumption of contaminated eggs, poultry meat, pork, or, less frequently, bovine meat (Velge et al., 2005; White et al. 2007; Collard et al., 2008). In addition to the public threat posed by *Salmonella*, it also has an economic impact on the poultry industry by threatening the domestic and export consumer markets and increasing the production and processing costs (Collard et al., 2008). Salmonellosis is a major public health concern and continues to have a serious economic impact on the Iranian poultry industry. Among all the serotypes of *Salmonella*, ser. Enteritidis is of particular concern because it is often found to be associated with clinical disease in humans (Velge et al., 2005; Gast, 2008).

In commercial poultry production, litter and dust are among the common sources of *Salmonella* contamination which may occur before, during or after the grow-out phase of production (Gast, 2008). In this study, therefore, *Salmonella* isolates from different sources with three origins mentioned above were examined (embryo mortality and day old chick before the grow-out phase, broiler, broiler breeder and layer feces during the grow-out phase, slaughter-

house after the grow-out phase). Due to public health concerns and the economic impact that the *Salmonella* contamination of poultry products may impose, it is of utmost importance for the industry to constantly investigate the most effective control strategies for *Salmonella* in poultry production.

The aims of the present study were to characterize a large collection of *Salmonella* isolates from different poultry sources with respect to antimicrobial resistance patterns, plasmid profiles, hemolysin production, and motility.

Materials and Methods

Bacterial isolates: A total of 123 *Salmonella* isolates from our bacterial collection in the Department of Avian Diseases, Faculty of Veterinary Medicine, University of Tehran were used in this study. All specimens had been collected during 2005-2007 from pullet, layer, and broiler flocks at different ages around the country. Our previous work on 123 *Salmonella* isolates determined that 70 (56.9%), 43 (35%), and 3 (2.4%) isolates belonged to serogroups D, C, and B, respectively. Seven isolates (5.7%) did not react to our antisera and remained unknown to us. All serogroup D isolates were found to be *S.* Enteritidis (Akbarian et al., 2012) (Table 1).

Drug susceptibility test: The susceptibility of the SE isolates to a panel of antimicrobial agents was determined by the agar disk diffusion method and the interpretation of results was carried out according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS, 2000). The antimicrobial agents that were tested and their concentrations (μg) were: ciprofloxacin (5), danofloxacin (10), ofloxacin (5), norfloxacin (10), enrofloxacin (5), levofloxacin (5), nalidixic acid (30), flumequine (30), cephalothin (30), ceftazidime (30), ceftriaxone (30), cefixime (5), ampicillin (10), amoxi-clav (30), carbenicillin (100), piperacillin (100), imipenem (10), kanamycin (30), neomycin (30), streptomycin (10), amikacin (30), gentamicin (10), tobramycin (10), lincospectin (15/200), chloramphenicol (30), florfenicol (30), furazolidone (100), tetracycline (30), and trimethoprim-sulfamethoxazole (1.25/23.75). All antibacterial disks were provided from Padtan Teb Co (Tehran, Iran). The ATCC reference strains *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa*, ATCC 27853, and *E. coli* ATCC 35218 were used for quality control purposes. In this study, the SE isolates with intermediate susceptibility classification were considered not to be resistant to that drug and the multiresistance was defined as resistance to more than one drug.

Plasmid profile analysis: A high pure plasmid isolation kit (Roche Applied Science, Mannheim, Germany) was used to extract and purify plasmid DNA from the bacterial isolates. Plasmids were separated by gel electrophoresis in 0.7% agarose gel in 1 x TAE buffer (Sambrook and Russell, 2001). The gels were run for 10 min at 100 volts and then approximately 2 hr at 70 volts, stained with ethidium bromide, exposed to ultraviolet light and photographed. Commercial DNA ladders (Fermentas, Germany) and an *E. coli* strain, AC11, containing three plasmids of 68 kb, 2.7 kb, and 1.7 kb (Peighambari et al., 1995) were used as molecular-weight markers in each gel running.

Hemolysin assay: All 123 isolates were cultured in 5% sheep blood agar (Difco, USA) with spot inoculation. Strains that showed biphasic lytic zone after incubation at 37°C for 24 and 48 hr were considered as positive (Peighambari et al., 1995).

Motility test: All 123 isolates were inoculated in tubes containing a pure motility medium (Bio Quest, USA) by stabbing the center of the column of medium to greater than half the depth and incubated for 24-48 hr at 35±2°C in an aerobic atmosphere (Quinn et al., 1994). Growth spread out from the line of inoculation if the organism was motile.

Results

Drug susceptibility test: All of 123 *Salmonella* isolates tested were susceptible to imipenem, norfloxacin, and ciprofloxacin (Table 2). The susceptibility to ceftriaxone, gentamicin, tobramycin, amikacin and ceftazidime was also very high. Resistance was predominantly associated with tetracycline, furazolidone, nalidixic acid, linco-spectin, and flumequine. Seventy-one resistance patterns to 29 antimicrobial agents were found among our 123 *Salmonella* isolates and 81% of *Salmonella* isolates were resistant to more than one antibacterial agent (data not shown). The resistance profile of 123 isolates to nine commonly used antibacterial agents in Iranian poultry industry

Table 1. List of *Salmonella* isolates used in this study. (1) All Serogroup D isolates belonged to serovar Enteritidis.

Isolate #	Serogroup1	Source
1-34	D	Embryo mortality
35	D	Hatchery environment
36-47	C	Broiler processing
48	B	Broiler processing
49-54	D	Broiler processing
55-60	C	Broiler processing
61-63	Unknown	Day old chick
64-74	D	Broiler
75-98	C	Broiler
99	D	Broiler
100-102	Unknown	Broiler
103-113	D	Broiler breeder
114	B	Broiler breeder
115-120	D	Layer
121	C	Layer
122	Unknown	Layer
123	B	Broiler processing

Table 2. Resistance of 123 Salmonella isolates to 29 antimicrobial drugs.

Antimicrobial drugs	% Resistant isolates
Ciprofloxacin (CP)	0
Danofloxacin (DFX)	5.69
Ofloxacin (OFX)	8.94
Norfloxacin (NOR)	0
Enrofloxacin (NFX)	24.39
Levofloxacin (LOM)	4.87
Nalidixic acid (NA)	43.08
Flumequine (FM)	40.65
Cephalothin (CF)	14.63
Ceftazidime (CAZ)	1.62
Ceftriaxon (CRO)	0.81
Cefixime (CFM)	9.75
Ampicllin (AM)	16.26
Amoxi-Clav (AMC)	5.69
Carbenicillin (CB)	4.06
Piperacillin (PIP)	4.87
Imipenem (IPM)	0
Kanamycin (K)	21.13
Neomycin (N)	22.76
Streptomycin (S)	39.02
Amikacin (AN)	1.62
Gentamicin (GM)	0.81
Tobramycin (TOB)	0.81
Linco-spectin (LP)	42.27
Chloramphenicol (C)	20.32
Florfenicol (FF)	10.56
Furazolidone (FR)	52.84
Tetracycline (TE)	66.66
Trimethoprim-Sulfamethoxazole (SXT)	32.52

(danofloxacin, enrofloxacin, flumequine, neomycin, lincospectin, chloramphenicol, florfenicol, furazolidone, tetracycline) varied and included 31 patterns (Table 3). Among 123 isolates, 24, 31, and 68 isolates, respectively, were susceptible, single resistant, and multi-resistant to the above mentioned nine agents.

Plasmid profile: Among 50 *Salmonella* isolates examined for their plasmid content, four plasmid profiles, A to D, were found (Table 4). In 23 (46%) isolates, no plasmid was detected and the other 54% of isolates contained one (38%) or three (16%) plasmids. The plasmid sizes ranged from 2.3 kb to 68 kb. The plasmids with sizes of 68 kb were the most frequent (Table 4).

Hemolysin assay and motility test: None of the 123 *Salmonella* isolates produced hemolysin but all isolates spread out from the line of inoculation and were motile.

Discussion

The most common serogroups of *Salmonella* isolates from poultry sources in our bacterial collection were D and C and all serogroup D isolates belonged to ser. Enteritidis (Akbarian et al., 2012). The dominance of ser. Enteritidis among *Salmonella* from poultry sources has also been documented by other researchers (van de Giessen et al., 2006, Snow et al., 2007, Snow et al., 2008). *Salmonella* Enteritidis has been one of the most common causes of food-borne infections in the last three decades (Velge et al., 2005). According to a report posted on Centers for Disease Control and Prevention (CDC) of the USA, SE ranked among the top two most frequently isolated serotypes from human sources reported to CDC in 2006 (http:/www.cdc.gov/ncidod/dbmd/phlisdata/*Salmonella*.htm).

Antimicrobial resistance in *Salmonella* is an important health concern in humans. Our data support the notion that antimicrobial resistance in *Salmonella* isolates from animal sources warrants close attention. To date, no clear evidence has been established that drug resistance and virulence are linked in *Salmonella*. However, the emergence of multidrug-resistant (MDR) *Salmonella* reduces the choices for antibacterial agents to combat invasive bacterial infections in both humans and animals. This study showed the presence of a significant number of MDR isolates among *Salmonella* that were recovered from various poultry sources. Our findings reinforced the need for continued monitoring of antimicrobial

Table 3. Drug resistance patterns among 123 *Salmonella* isolates to nine commonly used antibacterial agents in Iranian poultry industry. For abbreviations refer to Table 2.

Pattern #	Resistant to	No. of Isolates (%)
1	TE	20 (16.2)
2	TE, LP, FR	11 (9)
3	All with the exception of DFX	11 (9)
4	FM	5 (4)
5	FR	5 (4)
6	All with the exception of DFX, FF	5 (4)
7	TE, FR	4 (3.2)
8	FR, FM	4 (3.2)
9	TE, FR, FM	4 (3.2)
10	TE, LP, FR, FM	3 (2.4)
11	All with the exception of N, FF	Each pattern included only two isolates (1.6%).
12	All with the exception of C, DFX, FF	
13	TE, FM	
14	TE, LP	
15	TE, N, LP, FR	
16	TE, NFX, LP, FR, FM	
17	LP	Each pattern included only one isolate (0.8%).
18	All with the exception of FF	
19	All with the exception of N	
20	All with the exception of NFX, DFX	
21	All with the exception of NFX, N, FF	
22	TE, DFX	
23	FR, FM, FF	
24	NFX, FM, FR	
25	TE, NFX, LP, FR	
26	TE, NFX, FR, FM	
27	TE, N, LP, FM, C	
28	TE, NFX, N, LP, FM	
29	TE, N, LP, FR, FM	
30	TE, NFX,LP, FR, C	
31	TE, FR, FM, FF, DFX, NFX, LP, N, C	

Table 4. Results of plasmid profiling of 50 *Salmonella* isolates.

Plasmid profile	Molecular weights of bands (bp)	% of total	% of Serogroups	
			C	D
A	68000	38	34.28	46.6
B	-	46	62.86	6.7
C	68000, 29500, 5200	14	2.86	40
D	68000, 29500, 2300	2	-	6.7

resistance among animal bacterial pathogens and the value of laboratory antimicrobial susceptibility testing as the basis to make decisions for clinical treatment. The findings of this study indicated the increasing significance of poultry as the reservoirs of emerging MDR serovars. In Germany, Malorny et al. (2003) showed that the poultry and poultry meat are the main reservoir for Quinolone-resistant strains. In our study, the resistance of *Salmonella* isolates to most of the Quinolones tested was low. For example, all isolates were susceptible to norfloxacin and ciprofloxacin, and relatively less resistant to danofloxacin, ofloxacin and levofloxacin. One reason for this observation might be the lesser usage of these antimicrobial agents in the Iranian poultry industry. However, the resistance to enrofloxacin, which is currently among the most commonly used antibiotics against avian colibacillosis in Iran, was very high.

Development of antibacterial resistant genes in humans as a result of drug residue in poultry products has been attributed to imprudent use of antibacterial agents in poultry industries (Schwarz et al., 2001). On the other side, fluoroquinolone resistance in *Salmonella* is still relatively uncommon compared to its frequency in other *Enterobacteriaceae*. It has been suggested that this situation could be the consequence of somewhat different flouroquinolone resistance mechanisms in *Salmonella* (Giraud et al., 2006). Studies has shown that the prevalence of *Salmonella*-resistant isolates to extended-spectrum cephalosporins such as ceftriaxone and ceftazidime ranged from 0 to 3.4% between different continents and increased progressively from year to year (Arlet et al., 2006). In this study, resistance to ceftriaxone and ceftazidime was 0.81% and 1.62% but resistance to older generation of cephalosporins such as cephalothin was 14.63%. Antibacterial resistance, especially to flouroquinolones can be transferred from farm animals to human by food chain. Therefore, it is necessary to perform antibacterial susceptibility tests prior to the administration of these drugs (Frye and Fedroka, 2007).

Plasmid profiles may be of value as epidemiologic markers and may also suggest the presence of virulence plasmids. Plasmids of *Salmonella* Enterica may vary in size from two to more than 200 kb (Rychlik et al., 2006). The best described group of plasmids are the virulence plasmids (50-100 kb in size), which have been demonstrated in serovars such as Enteritidis, Typhimurium, Dublin, Choleraesuis, Gallinarum, Pullorum, and Abortusovis (Libby et al., 2004). The virulence plasmids encode for genes that are important in the disease producing ability of *Salmonella*. Another group of high molecular weight plasmids is the group of plasmids responsible for antibiotic resistance. The low molecular weight plasmids are the last group of plasmids found in S. enterica. We found four plasmid profiles among the *Salmonella* isolates examined in this study. Plasmid profiles may vary among *Salmonella* isolated from different geographic locations. However, many

studies have reported one large plasmid above 50 kb in most *Salmonella* isolates (Ang-Küçüker et al., 2000; Chu and Chiu, 2006; Rychlik et al., 2006; Avsaroglu et al., 2007; Morshed and Peighambari, 2010). There was one 68 kb plasmid in 54% of our isolates in different serogroups. In our previous study, we reported the same plasmid size in 97% of S. Enteritidis isolates (Morshed and Peighambari, 2010) and in this study, it was observed in 93.3% of S. Enteritidis isolates. The remaining 6.7% of SE isolates did not show any plasmid. In serogroup C isolates, the number of isolates that lacked any plasmid was much higher (62.86%) compared with those of SE isolates. Most serovars of *Salmonella* Enterica subspecies enterica do not possess any plasmids. Serovars such as Typhi, Paratyphi, Hadar, Infantis and most of the exotic serovars are usually free of any plasmids, although this notion is not valid for those isolates which are frequently recovered from infections of humans and farm animals. The reason for this remark is the observation that the isolates of these serovars possess serovar specific virulence plasmids typically 50-100 kb in size. In our study, 46% of all *Salmonella* isolates had no plasmid, which may be due to their origination from the healthy birds not the diseased ones. We also observed the 68 kb plasmid in 54% of all *Salmonella* isolates. This plasmid could be the serovar specific virulence plasmids in serotype Enteritidis. There were low molecular weight plasmids with sizes below 20 kb in 16% of our isolates. In *Salmonella*, the low molecular weight plasmids are found only in about 10% of *Salmonella* field strains and their biological functions are largely unknown (Rychlik et al., 2006).

Motility is one of the virulence factors in some bacteria (Brogden et al., 2000). Motility may increase the chance of bacteria coming into contact with the epithelial cells. The role of motility in the pathogenicity can be detected by cell invasion assays. Most *Salmonella* are motile with exceptions of *S*. Pullorum and *S*. Gallinarum. The roles of flagella in virulence of *Salmonella* are still not very clear. However, it is argued that the role of flagella may vary with the species of *Salmonella* and even with the disease model that they cause. Motility appears to be required for *S*. Typhi but not *S*. Typhimurium to invade epithelial cells (Clarke and Gyles, 1993). No role in pathogenicity was observed for the felagella in septicemic model of *S*. Typhimurium infection in mice, in contrast to the demonstration of the flagella role in enteric disease of *S*. Typhimurium in calves (Schmitt et al., 2001). As it was expected, all *Salmonella* isolates of the present study were shown to be motile.

Hemolysin has been proposed as a virulence factor in invasive bacteria causing infections in humans and animals. It may contribute to disease by making iron available to the bacteria from lysed red blood cells, or by damaging tissues (Brogden et al., 2000). One type of hemolysin purified from *Salmonella* was able to cause rapid lyses in Vero cells and several other cell lines (Clarke and Gyles, 1993). A previous study on 173 *Salmonella* isolates from poultry and human sources did not observe any hemolytic activity (Carramiñana et al., 1997), which is in accordance with findings of this study on *Salmonella* from poultry sources

In summary, various characteristics of a large collection of *Salmonella* strains from different poultry sources were determined in the present study. The resistance to antimicrobial agents among the *Salmonella* isolates was variable and MDR type was very common. Plasmids were found in 54% of isolates and in all of these plasmid bearing isolates, a large plasmid (68 kb) was shown. Information obtained from this study can be employed in future epidemiological studies.

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مطالعه خصوصیات جدایه‌های سالمونلا بدست آمده از منابع گوناگون مرتبط با طیور در ایران

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چکیده

زمینه مطالعه: سالمونلوز یکی از مهم‌ترین بیماری‌های مشترک انسان و حیوانات در سراسر جهان است. **هدف:** هدف از این مطالعه بررسی خصوصیات تعداد زیادی از جدایه‌های سالمونلا بدست‌آمده از منابع گوناگون مرتبط با طیور در ایران بود. **روش کار:** تعداد ۱۲۳ جدایه سالمونلا از منابع گوناگون طیور از نظر حساسیت داروئی، توانائی حرکت، تولید همولیزین، و الگوی پلاسمیدی (۵۰ جدایه) مورد ارزیابی قرار گرفتند. **نتایج:** تعداد ۷۱ الگوی مقاومت داروئی به ۲۹ عامل ضد میکروبی در بین ۱۲۳ جدایه سالمونلا یافت شد که ۸۱٪ از جدایه‌ها به بیش از یک عامل ضد میکروبی مقاوم بودند. الگوی مقاومت داروئی ۱۲۳ جدایه سالمونلا به ۱۰ ترکیب ضد میکروبی رایج در صنعت طیور ایران نیز متنوع بود و شامل ۳۱ الگوی مقاومت بود. در بین ۵۰ جدایه سالمونلا تعداد چهار الگوی متفاوت پلاسمیدی یافت شد. میزان ۵۴٪ جدایه‌ها دارای یک الی سه پلاسمید بودند که وزن ملکولی تقریبی آنها بین ۲/۳Kbs تا ۶۸ متغیر بود. در ۴۶٪ جدایه‌ها پلاسمیدی مشاهده نشد. در تمام ایزوله‌های دارای پلاسمید، یک باند ۶۸ کیلو بازی مشاهده شد. همه جدایه‌ها متحرک بودند اما تولید همولیزین در هیچ جدایه‌ای مشاهده نشد. **نتیجه‌گیری نهائی:** فراوانی مقاومت به عوامل ضد میکروبی در بین جدایه‌های سالمونلای پرندگان از نظر بهداشت عمومی حائز اهمیت فراوانی است.

واژه‌های کلیدی: سالمونلا، حساسیت داروئی، الگوی پلاسمیدی، طیور

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