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Characterization of the *Salmonella* Isolates from Backyard Chickens in North of Iran, by Serotyping, Multiplex PCR and Antibiotic Resistance Analysis

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ABSTRACT

The present study was designed to investigate the prevalence of *Salmonella* species, their molecular characterization and antibiotic resistance in backyard chickens. A total of 1125 samples were collected from backyard chickens in two consequent samplings. In the first part, samples were included of 820 poor recently hatched chicks, hatching residuals, egg shells in the nest floor, cloacal swabs and fresh litter droppings in the villages which located in north of Iran. Secondly, 305 samples were taken from newly hatched-chicks which fertile eggs were obtained from the rural chickens of those regions and incubated in laboratory incubator. Of 1125 samples tested, 27 (2.4 %) *Salmonella* were isolated that identified as serovars *of Salmonella* enteritidis (55.5 %), *Salmonella* typhimurium (22.2 %), *Salmonella* hadar (14.8 %) and *Salmonella* infantis (7.4 %). Except the traditional serotyping that was performed for all isolates, *Salmonella* typhimurium and *Salmonella* enteritidis isolates were characterized by using multiplex PCR for further identification. All of six Typhimurium serovars were positive for *rfb*J, *flj*B, *inv*A and *fli*C genes. In the case of Enteritidis serovars, polymerase chain reaction generated amplification products for *spv*, *sef*A and random sequence (specific for the genus *Salmonella*) in all of fifteen samples. Most of the *Salmonella* isolates in this study were sensitive to norfloxacin.

Keywords: Backyard chickens, *Salmonella*, Multiplex PCR

INTRODUCTION

Meat and poultry products are recognized as the major sources for transmitting *Salmonella* species to human with 40 % of the clinical cases attributed to the consumption of egg and poultry products (Sanchez *et al* 2002). In addition, many serovars of *Sarmonella* can produce serious diseases and death in chickens themselves especially at young age (Douglas- Waltman *et al* 1998). Raising backyard chickens are very common in rural and suburban regions in Iran, especially northern provinces same as similar situation in other countries. Every rural family in these provinces has usually an average of 50-100 domestic birds including chickens, turkeys, ducks and geese. It is estimated that approximately 20 millions of domestic birds consist of different species are produced annually by 500 thousand family in the northern provinces (Personal Communication, IVO). Backyard chickens are owned by the individual households and are maintained under scavenging system. Infected birds intermittently excrete *Salmonella*, a major zoonotic

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pathogen for the animals and humans into the environment which can result in cycles of *Salmonella* infection within flocks (Permin *et al* 1997, Sanchez *et al* 2002). Rural chickens are very important economically for their owners and additionally the health of rural people is influenced by the health status of their backyard poultry (Spradbrow 1990, Ellis 1992). Therefore it is necessary to determine the prevalence of *Salmonella* species in these flocks. Identification of *Salmonella* can be performed by both traditional serotyping and molecular methods. Serotyping offers a reliable method for differentiating *Salmonella* strains, but this procedure is usually depend on using a complete set of antisera and thus can be time-consuming. Several molecular methods based on the amplification of DNA have been developed for the detection of *Salmonella* serotypes (Jamshidi *et al* 2009; Herrera-Leon *et al* 2007, Tzu-Ming & Yi-Ju 2002). The main objective of this study was to investigate the prevalence of *Salmonella* in backyard chickens in the North region of Iran and to characterize the obtained isolates by traditional serotyping, multiplex PCR and antibiotic resistance analysis.

Materials and Methods

Sample collection in the villages. During the period from Jun 2006 to April 2007, a total of 820 samples from backyard chickens that were including of 219 poor newly hatched chicks, 200 different hatching residuals consist of egg shells and fluffs, 203 cloacal swabs of mature chickens and 198 droppings of the nest floor were taken in the different villages located from different regions in the north of Iran.

Sample collection from laboratory incubator. 250 fertile eggs of rural chickens were obtained from different villages and incubated under the controlled environment in the laboratory incubator located in the faculty of veterinary medicine, university of Tehran, until chicks hatched. In this case, 198 newly hatched chicks, 52 dead chicks or non hatched eggs, 19 pool fluffs and 36 pool residual of egg shells were collected for bacteriological studies and *Salmonella* isolation.

Isolation and identification of Salmonella. The procedure for the isolation of *Salmonella* was performed as described before (Douglas- Waltman *et al* 1998). Cloacal swabs and droppings as well as naturally hatching residuals were put into separate plastic bags, cooled in an icebox and immediately transported to laboratory. Poor chicks were euthanized in the laboratory and visceral organs (digestive tract, heart, liver and yolk sac) were removed and minced by using of sterile scissor. All collected samples were cultured in selenite F broth and incubated at 37 ℃ for 18 hr. Each sample then was inoculated onto *Salmonella- Shigella* agar (SS) and Brilliant Green agar (BG) plates. The plates were incubated at 37 ℃ for 24 hr. Suspicious colonies morphologically similar to *Salmonella* were subcultured for biochemical examinations. Biochemical characteristics were performed on Triple Sugar Iron (TSI) agar, Urea agar, Lysine-Iron Agar (LIA), Simon Citrate agar, motility medium and lactose, saccharose, maltos and mannitol broth.

Serotyping. *Salmonella* isolates from backyard chickens were used for serotyping. The *Salmonella* isolates were first cultured into TSI slant medium and grown overnight at 37˚C, then were tested by using antisera O (B, D, E, C) and H based on slide and tube agglutination tests to determine O and H antigens respectively (Douglas- Waltman *et al* 1998).

Multiplex PCR

DNA extraction and amplification. Prior to DNA extraction, *Salmonella* isolates from backyard chickens, were cultured onto Luria Bertani (LB) agar plates and incubated at 37°C for 24 h. For DNA extraction, one loopful of each sample from LB agar was suspended in 250 µl sterile distilled water. In order to have uniform turbidity, the

samples were vortexed, then were boiled for 10 min and centrifuged at 6000 ×g for 7 min. Supernatants were collected and saved for the multiplex PCR analysis. Multiplex PCR was performed (Jamshidi *et al* 2009) with two independent sets for DNA amplification of two serovars, Typhimurium and Enteritidis as described before. Briefly, four sets of primer pairs which are specific for *rfb*J (663 bp), *flj*B (526 bp), *inv*A (284 bp) and *fli*C (183 bp) in the case of *Salmonella* typhimurium (Table 1), and three sets of primer pairs which are designed for random sequence specific for genus *Salmonella* (429 bp), *sef*A (310 bp) and *spv* (250 bp) for *Salmonella* enteritidis, were used (Table 2). The amplification products were analyzed by agar gel electrophoresis. Electrophoreses of amplification products was on 1.2 and 1.8 % agarose gel for *Salmonella* typhimurium and *Salmonella* enteritidis sample respectively. In both reactions, 100 bp ladder was used as molecular weight marker. The gels were stained with ethidium bromide (2 µg/ml) to visualize fluorescent bands while using UV in the gel document system (BIORAD).

Antibiotic Resistance Analysis. The antimicrobial susceptibility of the *Salmonella* isolates was determined for 8 antibiotics including florfenicol (Ff), flumequine (Fm), ampicillin (Amp), lincospectin (Ls), tetracycline (Tt), neomycin (N), norfloxacin (Nfx) and sultrim (Sul) by using the disk diffusion method described by National Committee for Clinical Laboratory Standards (CDC 2003). Each *Salmonella* isolate was primarily cultured in Triptose Soy Broth (TSB) and incubated at 37 ˚C for a few hours then was calibrated with 0.5 Mc Farland $BaSO_4$ turbidity standards. Adjusted turbidity sample was then transferred to Mueller Hinton agar.

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	

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

Antimicrobial disks were disposed on the surface of inoculated agar media aseptically and incubated at 37 ˚C for 18-20 hr. Growth inhibition zone's diameter for each disk was measured and the results were interpreted with the compare to standards.

They were included of 15 *Salmonella* enteritidis (55.5%), 6 *Salmonella* typhimurium (22.2%), 4 *Salmonella* hadar (14.8%) and 2 *Salmonella* infantis (7.4%). Surprising results were also found in which all 6 *Salmonella* isolates from hatchery belonged to

Table 3. Distribution of Salmonella isolates among the samples collected in the villages or from laboratory hatched chicks

	Samples	Samples Nr	Positive Nr	Percentage of isolates (%)
Village	Newly hatched chicks	219	4	1.8
	Hatching residuals	200	2	1
	Cloacal swabs	203	6	3
	Litter droppings	198	9	4.5
Laboratory hatched chicks	Newly hatched chicks	198	1	0.5
	Non hatched chicks	52	0	0
	Chick fluffs	19	3	15.7
	Egg shells	36	2	5.6
	Total	1125	27	2.4

Table 4. The results of antibiotic resistance analysis in conjugation with different Salmonella serotypes isolated from villages and laboratory hatched chicks

Serotype	Isolates In village (n)	Resistance profile* In hatchery (n)	(%)	Resistant (n)	Resistance profile*
Enteritidis	9	6	55.5	14	Ls/Amp/Ff/Tt/ N/Sul/ Fm
Typhimurium	6	-	22.2	6	Amp/ Sul/ Tt/ Ls/ Fm/ Ff/
Hadar	4	-	14.8	3	Tt/ Amp/ Ff/
Infantis	2	-	7.4	2	Sul/ N/ Tt/ Ls/ Amp/
Total	27	100	25		

*Antibiotics are defined in text

RESULTS

Of 1125 samples which were taken, cumulatively 27 (2.4 %) *Salmonella* isolates were obtained (Tables 3 & 4). In the villages, isolates were associated with newly hatched chicks 1.8 % (4/219 chicks), naturally hatching residuals 1 % (2/200 samples), cloacal swabs 3 % (6/203 samples) and litter droppings 4.5 % (9/198 samples). The remaining of 6 isolates identified from the eggs hatched in the laboratory, were included of newly hatched chicks 0.5 % (1/198 chicks), pool chick fluffs 15.7 % (3/19 samples) and pool egg shells 5.6 % (2/36 samples). But no Salmonellae isolated from non hatched eggs (Table 3). Overall, of these isolates, four *Salmonella* serovars were identified. *Salmonella* enteritidis exclusively. The PCR produced 663, 526, 284 and 183 base pair amplification products from *rfb*J, *flj*B, *inv*A and *fli*C respectively in all of six *S.* Typhimurium serovars (Fig 1A). In the case of *S.* Enteritidis, PCR amplified 429, 310 and 250 base pair products from random sequence (specific for the genus *Salmonella*), *sef*A and *spv* in all of fifteen samples (Fig 1B). The results of antibiotic resistance analysis showed that most of the *Salmonella* isolates were sensitive to norfloxacin (93 %), flumequine (74 %) and florfenicol (70 %). Some of the isolates had intermediate sensitivity to neomycin, Sultrim and tetracycline, while the maximum antibiotic

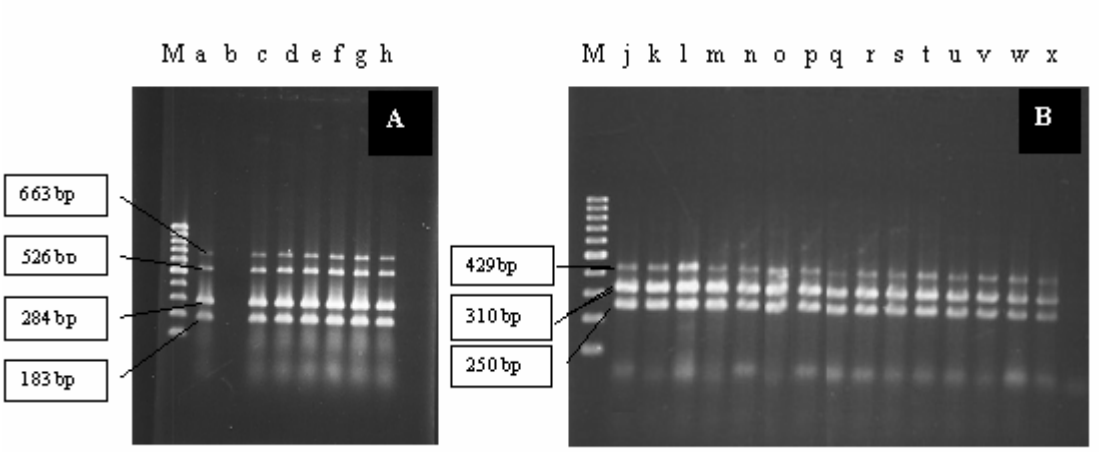

Figure 1. Multiplex polymerase chain reaction for detection of S. Typhimurium (A) and S. Enteritidis (B). Lane M: 100 bp ladder. Lane a: positive standard for S. Typhimurium. Lane B: Blank.Lanes c-h: ST isolates. Lanes j-x: S. Enteritidis isolates.

resistance was observed against ampicillin and lincospectin (63 %), (Table 4).

DISCUSSION

Village poultry are a neglected resource in many developing countries, where scavenging chickens of indigenouse breeds are kept by most rural households (Awan *et al* 1994). Under most village conditions, chickens are not optimally productive, but they do provide meat and eggs, that are very important local sources of protein. Ellis (1992) pointed out that with unique socio-economic conditions, high population densities, enormous numbers of small farms and farm-based village communities, small scale poultry farming is still very important in the Asian and African countries. In these countries, up to 80 % of the poultry population is kept by the households as free- range chickens (Aini 1990). Hence improvement in disease control and husbandry could greatly increase the productivity of village chickens (Spradbrow 1990). According to investigation of Bouzoubaa *et al* (1992) up to 58 % of the village chickens were seropositive against *Salmonella gallinarum/pullorum* in Morocco while, in Tanzania 2 % of these chickens had antibodies against *Salmonella enteritidis* (Permin *et al* 1997). In the present study 2.4 % of total samples-taken from backyard chickens were positive to *Salmonellae* enteritidis (55.5 %), typhimurium (22.2 %), hadar (14.8 %) and *Salmonella* infantis (7.4 %). The bacteriological survey by Jafari *et al* (2007) indicated that 5.8 % of pooled samples-taken from backyard flocks in south of Iran (Ahvaz) were positive to *Salmonella* serovars Typhimurium and Enteritidis. Such different variation between the south and north of Iran, could be due to differences in environmental contamination, health control programme, management systems and/or the sensitivity of procedure in two studies. Poppe (2000) reported that the serovar Enteritidis has replaced *Salmonella* typhimurium during the last 10-15 years as the commonest serotype in poultry, in many countries. This was in agreement with our study, because the predominant isolates in the native chickens from north of Iran were belonged to the serovar of *Salmonella* enteritidis. Such phenomenon could also be observed in our industrial poultry (Jamshidi

et al 2009, Morshed *et al* 2009, Bozorgmehri Fard 1991 and personal communication), however the study of Jafari *et al* (2007) resulted to isolate four serovars of *Salmonella* typhimurium and one *Salmonella* enteritidis from 300 backyard hens in south of Iran. Backyard chickens can be infected through contact horizontally e.g by wild animals especially wild birds. In the recent study of Mirzaie *et al* (2009) *Salmonella* serovars Typhimurium, Enteritidis and Montevideo were isolated from 3.8 % of the house sparrows which were captured partly from the rural area in the North of Iran. So, the isolated of these serotypes, particularly serovar Enteritidis, can indicate an important risk for contaminating of backyard chickens as same as local industrial poultry with *Salmonella* enteritidis. *Salmonella* was isolated not only from samples were obtained in the villages, but also from samples were taken from chicks that hatched in laboratory incubator. Isolation of *salmonella* from chicks-hatched in the hatchery indicated of vertical transition of germ from hens to fertile eggs (Heyndrickx *et al* 2000). The results of antibiotic resistance analysis showed that more than 90 % of the *Salmonella* isolates were sensitive to norfloxacin. Bacterial sensitivity to flumequine and florfenicol was also high as these antimicrobials are not commonly used in backyard chickens. On the other hand, most of the isolates had intermediate sensitivity or resistance to antibiotics such as neomycin, tetracycline, ampicillin and lincospectin which are mostly used in industrial poultry. While, backyard flocks are not treated with antibiotics as are consumed by the industrial poultry, such high percentage antibiotic resistant strains observed in this study could be due to transmittion of resistant bacteria from industrial poultry by human, free flying birds, poultry product and etc. Moreover, table 4 showed that 92.6 % (25 of 27) of the *Salmonella* isolates were resistant to at least one antibiotic. Unwise use of antibiotics in livestock and poultry with incorrect dose and duration or uncertain etiology, have been provoked the emergence of resistant strains in the nature. This could raise some concerns in the efficacy of human antimicrobial therapy, because resistant *Salmonella* strains can be introduced into human food chain and thus produce resistant infections. Our results also showed the correlation between traditional serotyping and PCR works. The specificity and sensitivity of the multiplex PCR make it potentially valuable tool for detection of *Salmonella* species. Thus molecular method can confirm traditional serotyping diagnostic that confirmed previous reports (Jamshidi *et al* 2009, Morshed *et al* 2009, Mirzaie *et al* (2009). Our investigation demonstrated that *Salmonella* could be transmitted both vertical and horizontally in backyard chicken, as in industrial poultry. Additionally, *Salmonella* characterization in such scavenging chicken operations is complicated because there are numerous potential sources of *Salmonella* contamination, since they are free in the villages. But they are an important *Salmonella* reservoir and may be a risk for human and industrial poultry therefore; an improvement strategy is needed for the study and control of *Salmonella* in these birds.

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