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Salmonellosis and Related Risk Factors in Broiler Flocks in Mazandaran Province, Northern Iran

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Abstract

Background: Prevention of foodborne pathogens is essential to control infectious diseases; *Salmonella* spp. is referred to as the most common causative agent of foodborne illnesses.
Objectives: The current study aimed to determine the prevalence of *Salmonella enterica* subsp. *enterica* in broiler flocks in Mazandaran province, north of Iran and find the potential risk factors including: age, size of flock, strain, season, vaccination program and use of antibiotics.
Materials and Methods: From March 2012 to December 2013, a total of 50 flocks were selected in slaughterhouse and 20 cloacal samples were collected from each flock. Every five samples were pooled and investigated for *Salmonella* spp. using polymerase chain reaction (PCR).
Results: Thirteen flocks out of 50 (26%) were positive for *Salmonella* species. Chances of *Salmonella* spp. detection was higher in flocks with lower age (P = 0.41). Increasing flock population was associated with increased chance of *Salmonella* spp. isolation (P = 0.21). The risk of salmonellosis in broiler flocks was increased when no antibiotics were given to day-old chicks. There was no significant difference (P = 0.30) in the prevalence of salmonellosis among different broiler strains.
Conclusions: In the current study, six risk factors were assessed for *Salmonella* spp. contamination in broiler flocks. Some of these factors contributed to the risk of salmonellosis in broiler flocks.

Keywords: Broiler Flocks, Risk Factor, Iran, *Salmonella* spp

1. Background

Prevention of food hazards in the first part of the food chain is essential to prevent illness of consumers. *Salmonella* spp. is cited as the most common causative agent of foodborne diseases (1). The genus *Salmonella* contains two species: *S. enterica* and *S. bongori*. Six subspecies are differentiated within *S. enterica* based on their biochemical and genomic characteristics, that one of them is *S. enterica* subsp. *enterica*. With regard to food safety, *S. enterica* subsp. *enterica* serogroups should be considered more than others, since they are known to cause 99% of *Salmonella* spp. infections in humans (2, 3). The usual route of infection in chickens is the oral uptake of *Salmonella* spp. from the environment. Contaminated food and water, for instance, are important sources of salmonellosis in chickens (4, 5). The vertical transmission of these bacteria can also be an important issue in poultry (6, 7). Different prevalence rate of these bacteria are reported between countries, nearly 0 in Sweden, 68.2% in Hungary (8), 76.9% in Canada (9); 69.8% in France (10); 41.3% in Turkey (11) and 25% in Denmark (12). Some management and environmental risk factors are associated with the incidence of salmonellosis in the flocks. The environmental persistence of *Salmonellae* spp. is a significant factor in the epidemiology of these bacteria in poultry by creating opportunities for horizontal transmission of infection within and between flocks (13). The role of rodents, flies, beetles and wild birds as vectors in *Salmonella* spp. transfer is extensively discussed (14-17). Stress has an immunosuppressive effect in egg laying hens, which can have negative consequences with respect to salmonellosis and shedding (18, 19). There are few studies on the role of vaccination programs and using antibiotics in rearing the period with salmonellosis.

2. Objectives

Mazandaran province in Northern Iran is one of the major poultry production areas. No study is conducted to determine the prevalence of salmonellosis in poultry flocks in this region. Therefore, the present study aimed to determine the prevalence of *Salmonella* spp. in broiler

flocks in Mazandaran province and its possible associations with some potential risk factors including age, size of flock, strain, season, vaccination programs, and use of antibiotics during the production period.

3. Materials and Methods

A cross-sectional study was conducted to determine the prevalence of salmonellosis and related risk factors in broiler flocks.

3.1. Sample Collection

A total of 1000 cloacal swab samples were randomly collected from 50 broiler flocks (20 swabs per each flock) on the slaughter line of four abattoirs of Mazandaran province from March 2012 to December 2013. Sampling was randomly done in a systematic manner, which means each cloacal swab was taken from one of each five birds in the slaughter line.

3.2. Data Collection

To evaluate potential risk factors, a questionnaire that structured the parameters such as: age, size of flock, strain, season, vaccination programs and antibiotics used in the rearing period was completed for each flock.

3.3. Enrichment of the Samples

Every five samples from each flock were pooled, and were pre-enriched in buffered peptone water in 1:10 sample/broth ratio at 37°C for 24 hours. One milliliter of this pre-enrichment broth was used to inoculate 10 mL of cysteine-selenite broth (Merck, Germany).

3.4. DNA Extraction

DNA extraction was performed through the protocol described by Khoshbakht et al. (20). One milliliter of each enriched fecal sample was transferred to a 1.5 mL micro-tube and centrifuged at 10,000 rpm for two minutes. Pellets were re-suspended in 570 μL of Tris-EDTA (TE) buffer (10 mM Tris-HCl pH = 8; 1 mM Na_2 ethylenediaminetetraacetic acid: EDTA), 30 μL 10% sodium dodecyl sulphate and 4 μL proteinase K (Fermentas, Germany) in a concentration of 20 mg/mL. Samples in micro-tubes were mixed vigorously before incubation at 56°C in a water bath for one hour. One hundred microliters of 5 M and One hundred microliters of CTAB/NaCl (cetyltrimethylammonium bromide: CTAB 10%, NaCl 0.6 M) were added and mixed. After incubation at 65°C in water bath for 12 minutes, 500 μL of phenol/chloroform/isoamyl alcohol (25:24:1) was added and vortexed. Then samples were centrifuged at 13,000 rpm for seven minutes. Five hundred-fifty microliters of supernatant was transferred to a fresh micro-tube and equal volume of chloroform/isoamyl alcohol (24:1) was added. The samples were mixed and centrifuged as above. The supernatant was transferred to a new micro-tube and 300 μL isopropanol was added to each tube, then centrifuged at 10,000 rpm for three minutes and the pellet was washed in 70% ice-cold ethanol and centrifuged again at 10,000 rpm for three minutes. The final pellet was re-suspended in 50 μL of TE buffer and stored at -20°C until polymerase chain reaction (PCR) was performed.

3.5. PCR Reaction

PCR amplifications were performed in a final volume of 25 μL. The PCR reaction mixtures consisted of 2 μL of the DNA template, 2.5 μL 10X PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $[NH4]_2SO_4$), 1 μL dNTPs (50 μM), 1 μL (1 U Ampli Taq DNA polymerase), 1 μL (25 pmol) from each forward and reverse primers (CinnaGen, Iran) in the total volume of 25 μL using distilled deionized water. The forward (S139) and reverse (S141) primer sequences were 5′GTGAAATTATCGCCGCCACGTTCGAA3′ and 5′TCATCGCACCGTCAAAGGAACC 3′ (21), respectively. The thermal cycler (MJ mini, BioRad, USA) was adjusted as follows: initial denaturation at 94°C for four minutes, followed by 33 cycles of denaturation at 94°C for one minute, annealing at 57°C for one minute and extension at 72°C for one minute. Final extension was carried out at 72°C for seven minutes. Previously identified *Salmonella* strains isolated from animals, in faculty of veterinary medicine of Amol, were used as positive control. Amplified products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide. Visualizations were undertaken using a UV transilluminator (BTS-20, Japan), and the 100 bp DNA ladder was used as molecular size marker.

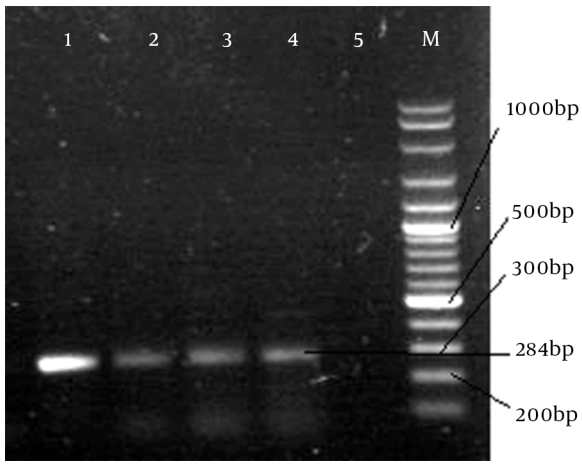
3.6. Statistical Analysis

Prevalence of infection and corresponding 95% confidence interval (CI) were estimated. Possible association between the contamination with *Salmonella* spp. in the flock and the risk factors was investigated using univariate and multivariate logistic regression analyses. Variables with P value equal or less than 0.30 were included in the multivariate logistic regression analysis. Final logistic model was fitted based on stepwise backward elimination procedure and significance of Wald statistics. A P value less than 0.05 was considered statistically significant in the final model.

4. Results

Of the 1000 cloacal samples analyzed by PCR, the prevalence of *Salmonella* spp. contamination in the broiler was 26%. Thirteen out of 50 flocks were positive for *Salmonella* species (Figure 1). Summary of statistics for broiler flocks variables according to the rate of contamination with *Salmonella* spp. are shown in Table 1.

Figure 1. PCR Final Product for Differential Detection of *Salmonella* Species



Lane 1, positive control; Lanes 2 - 4, positive samples; Lane 5, negative control; Lane M, DNA size marker.

Table 1. Summary of Statistics for Broiler Flocks Variables According to the Rate of Contamination With *Salmonella* spp.[a]

Variable	Positive Flocks (N = 13)	Negative Flocks (N = 37)	P Value
Age at slaughter, d	52.6 ± 6.1	58.1 ± 3.6	0.41
Flock size (bird)	15245 ± 7840	10231 ± 4551	0.21

[a]Values are expressed as mean ± SD.

Age of flocks at slaughtering time was lower in positive flocks, but not significant (P = 0.41). The mean of bird population in negative flocks was slightly lower than that of the positive ones, but the difference was not statistically significant (P = 0.21). One or more kinds of antibiotics were used in nearly 90% of the flocks during the rearing period, and enrofloxacin was used most widely. According to the statistical analysis, there was no relationship between the use of enrofloxacin and isolation of *Salmonella* spp. (P = 0.17); but, the risk of salmonellosis in broiler flock was increased when no antibiotics were given to day-old chicks (P = 0.04). All of the studied broiler flocks had been vaccinated against avian influenza virus (AIV), Newcastle disease (ND), and infectious bursal disease (IBD) according to the regional vaccination program; however, only 45% received infectious bronchitis virus (IBV) vaccine. Surprisingly, the isolation of *Salmonella* spp. was insignificantly higher in flocks which received IBV vaccines (P = 0.23). There was no significant difference (P = 0.30) among different strains (Ross, Cobb, and Arbor acres) regarding the contamination with *Salmonella* spp. According to the season, the frequency of *Salmonella* spp. isolation were higher and lower in flocks sampled in winter (17.8%) and in spring (9.2%) compared to those of autumn and summer (7.1% and 6.9%, respectively); but differences were not statistically significant (P = 0.09) . Four variables including seasons of sampling (P = 0.09), broiler strains (P = 0.30), using antibiotic on day one (P = 0.04) and vaccination against IBV (P = 0.23) were included in the logistic model (Table 2). Results showed that odds of infection increased with using antibiotic on day 1 (P = 0.04) and vaccination against IBV (P = 0.23).

Table 2. Results of the Final Logistic Regression Model for Contamination With *Salmonella* spp. in Broiler Flocks in Iran

Variable	P Value	Standard Error	Odds Ratio
Season	0.09	1.41	9.1
Strain	0.30	1.01	15.3
Use of antibiotics on day one			
Yes	NA	NA	1
No	0.04	0.89	7.1
Vaccination against IBV			
Yes	0.23	1.77	7.23
No	NA	NA	1

Abbreviations: IBV, infectious bronchitis virus; NA, not available.

5. Discussion

Overall, thirteen out of 50 flocks (26%) were positive for *Salmonella* species. This finding was in agreement with the results of Chadfield et al. (12), and Gutierrez et al. (22) who reported 25% in Denmark and 27% in Ireland, respectively. Ansari-Lari et al. (23) reported that 22.5% of the broiler flocks of Shiraz were positive for *Salmonella* spp. The difference observed between the results of the current study and those of the latter study may be due to different detecting procedures; they used culture methods. Excretion of *S. enterica* decreases with age (24). In the current study,

the positive flocks had lower age, but the age of the chickens was not a significant variable in the present investigation. Skov et al. (25) showed that the risk of salmonellosis increased when the flock size was larger, which is in line with results of the current study. In the current study, the prevalence of *Salmonella* spp. was the highest (17.8%) in winter and the lowest (6.9%) in summer. These results contradict with those of the studies by Bouwknegt et al. (26), Mollenhorst et al. (27), Namata et al. (28) and Huneau-Salaun et al. (29); but in accordance with those of Wales et al. (30) and Upadhyaya et al. (31) that reported a seasonable effect in their studies. The chance of *Salmonella* isolation in flocks sampled in winter was significantly higher than that of the flocks sampled in the other seasons. A high density of animals is a well-known risk factor for contamination with *Salmonella* spp. (8, 29). Furthermore, the air quality in flocks seems to be lower in winter (32, 33). This can cause stress in broilers, leading them from a Salmonella-carrying state to a Salmonella-shedding state. The results showed no relationship (P = 0.30) between broiler strain and salmonellosis, which were in agreement with the results obtained by Skov et al. (25) and Huneau-Salaun et al. (29). According to the results, using antibiotics in day-old chicks reduces the chance of *Salmonella* spp. isolation. This finding was supported by the results of another study (11). Prophylactic usage of antibiotics against mortality during the first day of life can reduce the number of colonized and shed bacteria (34-36). In Iran, there are different opinions among farmers about the use of IBV vaccine. Although IBV vaccination is used in a nationwide program in Iran, a significant proportion of farm owners do not use IBV vaccination in their flocks due to their undesirable personal experience with this vaccine in the field (35). Results of this study showed an association between IBV vaccination and salmonellosis. Volkova et al. (36) showed that increased dosage of IBV vaccine delivered via spray to the one-day-old birds was linked to a higher probability of *Salmonella* spp. isolation from the flock, which is in line with the current study findings.

In the current study, six risk factors were assessed for contamination with *Salmonella* species in broiler flocks. This is the first time that such results are obtained in Iran. Further studies on *Salmonella* serotypes during rearing period are necessary.

Footnotes

Authors' Contribution: All authors contributed equally in this article, especially in design, laboratory analysis, statistical analysis and manuscript writing.

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