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RESEARCH ARTICLE

Association between Poultry Density and *Salmonella* Infection in Commercial Laying Flocks in Iran using a Kernel Density

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ABSTRACT

Salmonellosis in laying flocks is one of the major health concerns worldwide and the size of the flock has been introduced as an important risk-factor associated with increased risk of *Salmonella.* In this study a total of 113 holdings was selected using simple random sampling, stratified by active layer holdings in each province of Iran. Two pooled fecal samples were obtained from each flock of holding and information of sampled holdings was acquired from Geographic Information System (GIS). The prevalence of *Salmonella* contamination in laying hen holdings was 3.5% and the risk of *Salmonella* contamination was associated with the size of the poultry-holding (OR=5.6; CI95%=1.35, 23.57; P=0.018). None of the positive farms were in high density surface at farm level. Two of the positive farms were at the density surface of more than 13 flocks per square kilometer and all the positive farms were in the regions with poultry density of more than 319,000 per square kilometer. According statistical and spatial analysis keeping large number of poultries in a certain area is an important risk factor for *Salmonella* contamination.

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INTRODUCTION

Salmonella is an important zoonotic foodborne pathogen worldwide. Salmonellosis in laying flocks is one of the major health concerns all over the world. Consuming poultry products, especially eggs are responsible for many of human zoonotic gastroenteritis (EFSA, 2009; Huang *et al*., 2016). It can cause a wide range of health problems from mild gastroenteritis to bacteremia and typhoid fever. It has been estimated that *Salmonella* annually causes 93.8 million cases of gastroenteritis and 155000 deaths (Majowicz *et al.*, 2010). *Salmonella* threat to public health results in considerable economic consequences in many parts of the world and in addition to the health consequences, *Salmonella* infection also has a severe economic impact (Collard *et al.*, 2008; Jing *et al.*, 2014). *Salmonella* contamination is very crucial for exporting the poultry products and the satisfaction of consumers.

Due to the importance of *Salmonella* contamination in laying flocks several studies have been carried out to detect risk factors at different levels of production systems (Bokaie *et al.*, 2016). Hatcheries and feed mills are potential sources of infection, environmental contamination and ineffective cleaning and disinfection between flocks persist the infection and rodents and arthropods carry over *Salmonella* between flocks and holdings (Snow *et al.*, 2010; Kanwal *et al.*, 2015). Housing in conventional cages, sampling in the winter and the presence of birds of different ages are factors which increase the risk of *Salmonella* contamination of holding (Van *et al.*, 2010; Srinivasan *et al.*, 2014).

The size of the flock has been introduced as an important risk factor associated with increased risk of *Salmonella* shows that holding flocks larger than 30,000 birds are 14.88 times more probable of being *Salmonella* positive (Snow *et al.*, 2010). In a study assessing 122 items for *Salmonella* contamination in laying farms, flock

size was one of the three main risk factors for *Salmonella* contamination (Huneau-Salaün *et al.*, 2009). However, we need to conduct more studies on the risk factors of *Salmonella*. The added evidence in this field can help us to establish more effective control programs for *Salmonella.*

Distance to the nearest farm is a factor that can interact with poultry density because when farms are nearer to each other, size of population at risk will increase in the unit area. It was indicated that more than 1 kilometer distance between farms can have a protective effect for *Salmonella* contamination in layers (Snow *et al.*, 2010).

Most of the previous studies have focused on analyzing risk factors and modeling multiple risk factors using statistical models, such as logistic regression (LR) models. In this study, we took into account all the farms in 1 square kilometer unit of area using smoothing techniques. In addition to usual statistical analysis, Kernel density function is used to identify *Salmonella* contamination pattern in layer flocks.

MATERIALS AND METHODS

Study design: This cross sectional study carried out between June 2013 and March 2014. For prevalence survey the target population was all commercial laying holdings in Iran producing table eggs, which are listed systematically every season by Iranian Veterinary Organization.

Sampling: The number of laying hen flocks for sampling was calculated with an expected holding prevalence of 5%, precision of 4% and a confidence limit of 95%. The sample was then divided regarding the proportion of the number of active holdings in each province. In each province, holdings to be sampled were randomly selected. The epidemiological unit of the study was a holding. The *Salmonella* contamination of the flocks was assessed by taking two pooled feces samples from each house of the holding. All the holdings with at least one positive fecal sample considered positive.

Independent variables: Information about potential risk factors of *Salmonella spp.* contamination of the flocks was obtained from the Iranian Geographic Information System (GIS). Data from each commercial flock is officially recorded in the GIS by poultry administrators of each province. We collected data concerning general characteristics of the holding and sanitary practice in the farm. Samples were sent to the province reference laboratory.

Bacteriological tests: Each fecal sample (25g) was added to Buffered Peptone Water (225mL, 25˚C) and incubated at 37˚C for 18 h. Then 1 mL of each diluted sample was transferred to 9 mL of Selenite-Sisteinbroth (S-S) and Rappaport-Vassiliadis (R-V) broths, and they were incubated (42˚C/24 h for R-V and 37˚C/24 h for S-S). At the next stage (Isolation), Xylose Lysine Desoxycholate (XLD) agar, MacConkey agar and Salmonella-Shigella (SS) agar as Differential-Selective media were used to detect probable *Salmonella* colonies (Plates were incubated at 37˚C for 24-48 h). Suspected colonies (colorless colonies on MacConkey and SS agars; pink colonies with black centers on XLD agar) were picked and transferred to Identification stage. In this stage, suspected colonies were cultured in Triple Sugar Iron agar, Lysine Iron agar, Urea agar, and tested by standard methods including IMVIC and Oxidase tests. For final confirmation, positive microorganisms in the former chemical tests underwent Serological (somatic and flagellar serotyping) and Molecular (PCR) tests in the Reference Laboratory of Veterinary Organization of Iran (Sikder *et al.*, 2005; de Freitas *et al.*, 2010; Lungu *et al.*, 2012; Bokaie *et al.*, 2016). We used *inv A* (Rahn, 1992) to identify *Salmonella, fli C* (Soumet, 1999) for *Salmonella typhimurium, flj B* (Kardos, 2007) for *Salmonella infantis* and *prot 6e* (Malorny, 2007) for *Salmonella entretidis.*

Spatial analysis: The information of all the sampled holdings was recorded and the 11 digit unique GIS codes of them were used to find x and y coordinates. Point pattern maps were created using the GIS arc map (ESRI-10.2) and Iran base map supplied by the National Cartographic Center of Iran (NCC).

The spatial point distribution of all selected holdings was assessed and then plotted by the Kernel method using GIS arc map (ESRI-10.2) (Carpenter, 2001). In our study Kernel technique was applied to determine smoothed density in three levels; density of holdings, density of houses and the density of poultries. In this study the normal method of interpolation was used with a fixed bandwidth of 1 kilometers and a cell size of 38.8 meters.

Risk factor analysis: Due to a small number of positive holdings rare events logistic regression was used for statistical analyses using STATA-12 (King and Zeng, 2001). A two-step statistical performed to assess the relationship between explanatory variables and the *Salmonella* contamination in layer flocks. In the first step, a univariable analysis carried out. Variables associated with *Salmonella* status ($P<0.20$) were then selected. Biological plausibility and co-linearity between variables were taken into account in the selection of variables that were retained.

RESULTS

Four of the 113 flocks were positive for *Salmonella spp.* The mean age of sampled flocks was 48.31±16.75 weeks. The obtained prevalence of *Salmonella* contamination was 3.5% (CI 95%=0.08, 6.9). Two of positive samples could not undergo sero grouping and two of positive samples belonged to B serogroup and one of them was *Salmonella typhimurium.* All the four positive samples underwent PCR test and according the PCR, it was confirmed that all of these samples are *Salmonella*. PCR test also performed for *Salmonella infantis*, *Salmonella typhimurium* and *Salmonella enteritidis*. The result was negative, so we were not able to identify the serotype of three positive samples.

In this study, seven variables were assessed and according the univariate analysis two of them were significant at $P<0.2$ and were included in the multivariable model (Table 1).

Table 2 shows the final multivariable model with adjusted ORs and 95 percent confidence intervals for selected factors at farm level.

Table 1: Definition and distribution of explanatory variables selected for the multiple logistic-regression model of risk factors for *Salmonella* contamination in laying hen holdings (113 holdings, Iran, 2013–2014)

Definition of variables	% of holdings	% of *Salmonella* positive holdings	p[a]
Poultry- size (no. of laying hens of holding)			
<150,000 laying hens	88.5	2.0	0.03
≥150,000 laying hens	11.5	15.4	
Number of houses of holding			
<6houses	81.4	2.2	0.06
≥25 houses	18.6	9.5	
Age (week)			0.94
<35	23.0	3.8	
35-70	51.3	3.4	
≥70	25.7	3.4	
Holding has damping equipment			-
Yes	96.0	4.12	
No	4.0	0	
Holding has fencing			-
Yes	89.2	4.4	
No	10.8	0	
Site all-in/all-out			0.55
Yes	72.6	3.6	
No	27.4	3.2	
Sampling season			0.91
Spring	7.1	12.5	
Summer	58.4	3.0	
Autumn	23.9	0	
Winter	10.6	8.3	

[a.]The probability associated with the variable in univariable rare event logistic regression model.

Table 2: Final mixed logistic-regression model of risk factors for *Salmonella* contamination of Iranian laying hen flocks (113 flocks, Iran, 2013–2014)

Variables	%*Salmonella* positive flocks	Logistic-regression model[a]	
		OR[b]	95% CI[c]
Poultry-size (no. of laying hens of holding)			
<150,000 laying hens	2	5.6	1.35-23.57
≥150,000 laying hens	15.4	1	
Number of houses of holding			
<6 houses	2.2	2.21	0.53-9.18
≥6 houses	9.5	1	

Intercept = -3.66, 8 (P<0.001); OR[b]: Odds Ratio. CI[c]: confidence interval.

The risk of *Salmonella* positivity increased when the size of the poultry-holding was greater than 150,000 laying hens (OR=5.6; CI95%=1.35, 23.57; P=0.018). The number of flocks of holding more than 6 (OR=2.21; CI 95%=0.53, 9.18; P=0.273) was also associated with a higher risk of *Salmonella* contamination. However, the latter was not statistically significant.

All the four positive holdings are allocated in the center of Iran. Fig. 1, 2 and 3 show the distribution of farms, and their surface density of farm, flocks and poultries respectively generated by the spatial point analysis (Kernel density). All the positive farms are located in the density surface of lower than 3 farms/km^2. There are two positive farms at the density surface of more than 12 flocks/km^2 and all the positive farms are in the regions with poultry density of more than 300,000 poultries/km^2.

DISCUSSION

This study shows that some of the commercial layer farms in Iran are contaminated with *Salmonella spp*. although the apparent prevalence is low. The *Salmonella* surveillance program has not been established in Iran and our knowledge about *Salmonella* contamination of layer flocks is limited to previous restricted studies. A study on *Salmonella* contamination in laying flocks carried out in 2010 in Tehran province of Iran revealed a *Salmonella* prevalence of 9% (Morshed and Peighambari, 2010). Although due to small sample size of this study (11 layer flocks) the precision of estimated prevalence is low. One study on zoonotic *Salmonella* in 250 retail eggs of Mashhad city of Iran during the summer of 2008 demonstrated that 1.6% of examining egg shells are contaminated with *Salmonella typhimurium* while none of the egg contents were *Salmonella* positive (Jamshidi *et al.*, 2010). At the same time, similar study carried out in Shahre-kord city of Iran on 100 retail eggs and all of them turned out to be *Salmonella* negative (Safaei *et al.*, 2012). It might be worth pointing out that 56.48% of 108 commercial layer flocks were positive for *Salmonella enteritidis* in a study performed using the ELISA test in 2008 while none of the *Salmonella* isolates in this study were *Salmonella enteritidis* (Akbarian *et al.*, 2009). It may be concluded that we need more sensitive study designs to reach more precise estimates of prevalence. Knowledge about human Salmonellosis cases in Iran is also limited because of the lack of reporting system in this case.

Using the statistical approach, the logistic regression analysis revealed the poultry size as an important risk factor, as also observed in other studies (Huneau-Salaün *et al.*, 2009; Snow *et al.*, 2010). It may be due to several factors; a. increasing the population at risk, b. More difficult cleaning and disinfection of houses, c. Stress of intensive production system which can increase the susceptibility of poultries.

Number of houses were significantly associated with *Salmonella* contamination in univariable analysis, but multivariable analysis revealed that poultry size has confounded this relationship and number of houses is not an important risk factor.

Using statistical techniques, we could assess risk factors at holding level, but we were not able to go further and take into account nearby farms characteristics. Therefore, we used "Kernel density" approach for smoothing the risk factors related to density. According to the Kernel smoothed map, the risk of poultry density could be even higher than which calculated by statistical tools. In our view the effects of poultry density in a certain area are being greater than those estimated by the models relying on just one holding characteristics.

This three Kernel smoothed maps also suggest this idea that keeping large number of poultries in a small number of holdings is an important risk factor for *Salmonella* contamination and it would be preferable to establish more holdings with lower poultries per holding. It probably can modify the effect of poultry density. This can help improve management and cleaning practices and will detach population at risk into smaller groups. According the results flock density is not an important factor for *Salmonella* contamination.

KD smoothing has been previously used for visualization purposes such as disease mapping and the detection of disease clusters (Siqueira *et al*., 2004; Lau *et al*., 2012; Si *et al*., 2013). In our study, we introduced a novel application for this analytical technique which may be used for epidemiological researches and finding new risk

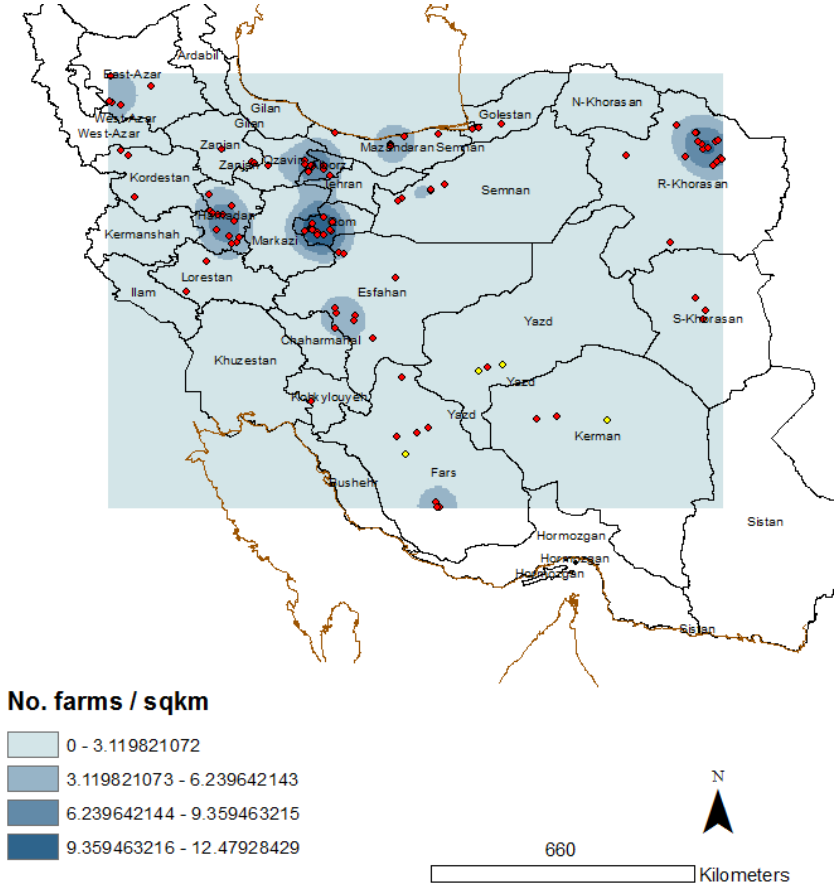

Fig. 1: Spatial distribution of positive and negative farms in Iran and their juxtaposed holding level density estimates (average number of holdings/km^2 in 2013-2014).

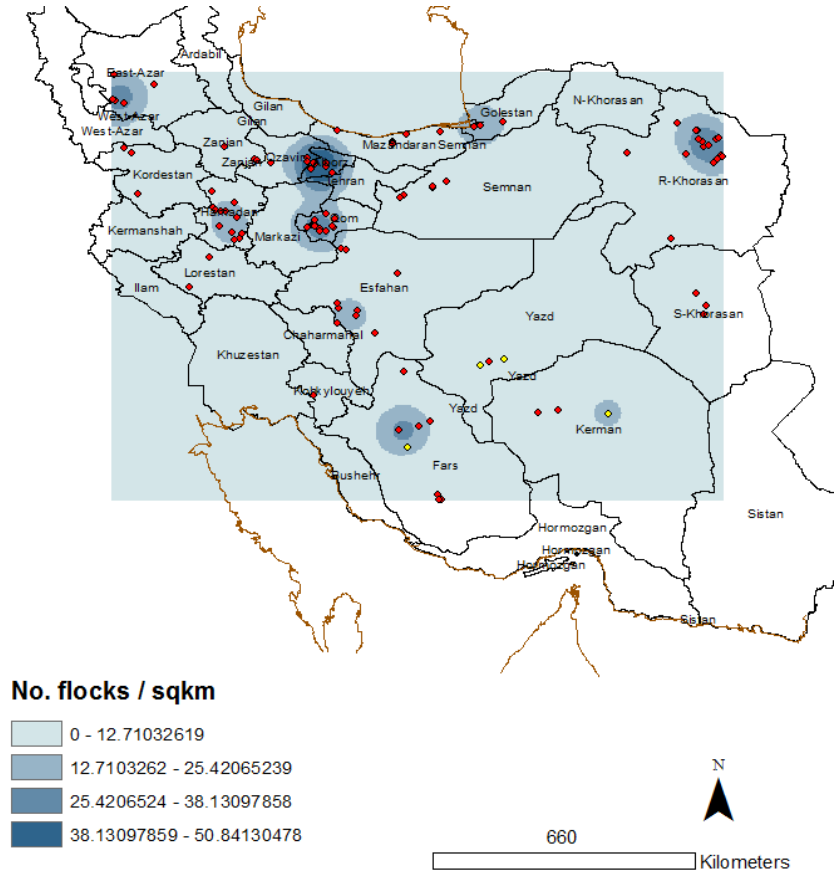

Fig. 2: Spatial distribution of positive and negative farms in Iran and their juxtaposed flock level density estimates (average number of flocks/km^2 in 2013-2014).

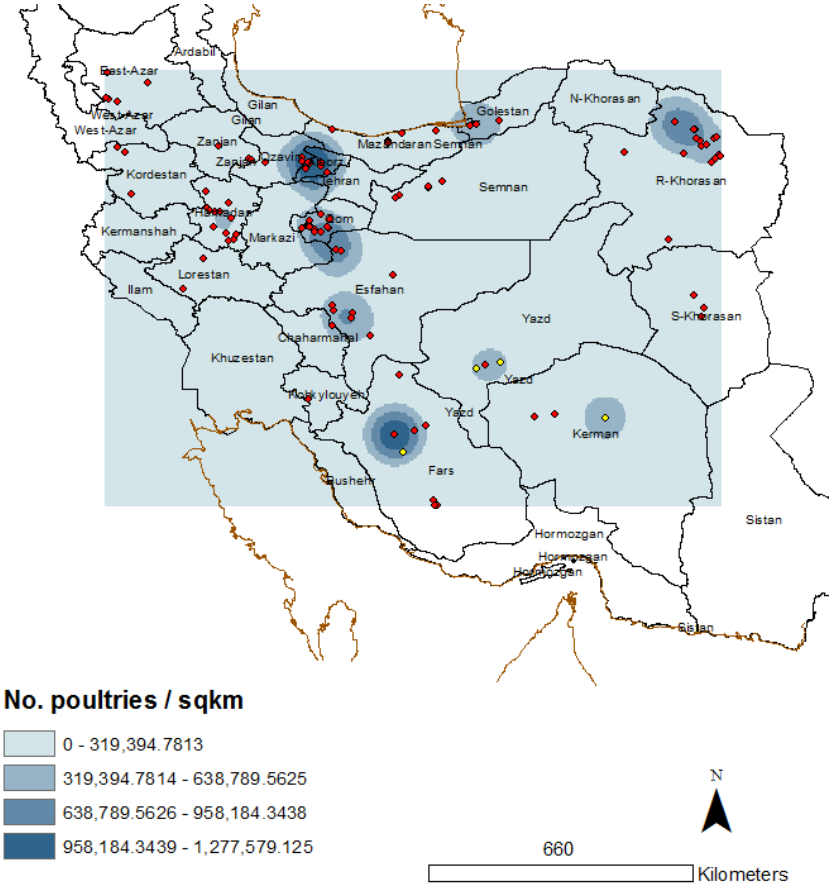

Fig. 3: Spatial distribution of positive and negative farms in Iran and their juxtaposed poultry level density estimates (average number of poultries/km^2 in 2013-2014).

factors for the health outcomes. Importantly, the KD technique can facilitate assessing risk factors which have a widespread effect in a certain area and may also interact with nearby features. Using this technique is a simple way for visualization risk factors and their effect on health events.

Conclusions: Poultry size is an important risk factor for *Salmonella* contamination in Iranian laying hens. The smoothed density maps suggest that poultry size not only is a crucial factor at the farm level but also is important at the area level. In other words, when there are several farms with high poultry density near to each other the chance of *salmonella* contamination may increase.

Authors contribution: FA, HP, SB, SMP, MM and MHF contributed to the design of the experiment. FA and HP analyzed the data, and wrote the manuscript. FT, AR, SAG and MS conducted the experiments.

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