Prevalence and risk factors associated with *Campylobacter* among layer farms

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Abstract

Campylobacter jejuni is an important food-borne pathogen. The main source of this pathogen is poultry and poultry products. Poultry farms of low biosecurity level plays major role in disseminating this pathogen. The objectives of this study were to investigate the occurrence of *Campylobacter* and identify potential risk factors associated with their presence in layer farms in Northern Jordan. A total of 2524 samples from chickens, litter, water and feed were collected from 35-layer farms. Samples underwent conventional and enrichment isolation methods for *Campylobacter*. Confirmation was done morphologically, biochemically and by PCR typing. The flock-level prevalence of *C. jejuni* was 40%, 37%, 20% in chicken cloacae, drinking water and litter respectively. *C. jejuni* was the only confirmed isolated species. None of the feed samples revealed presence of *Campylobacter*. The concentration of free residual chlorine was below the recommended standard levels. The risk factors were identified using modified semi-structured questionnaire. There was no significant association between evaluated risk factors and isolation status potentially reflecting small number of study farms. The prevalence rate for *C. jejuni* is within commonly reported range. High stocking density, short distance between farms, improper hygienic practice and low water chlorine level seems to increase occurrence rate of *Campylobacter* in layer farms. Educational biosecurity programs regarding *C. jejuni* transmission and their public health importance needs to be established.

Introduction

Campylobacter organisms are gram negative spiral-shaped bacteria which inhabit the intestine of many species and can cause clinical manifestation of variable severity.[1] *Campylobacter* is considered of great public health significance worldwide because it is the most reported gastrointestinal bacterial foodborne pathogen.[2] Most commonly reported human illnesses are caused by *Campylobacter jejuni*.[2] In the United States, there have been reports of more new 2 million *Campylobacter* cases per year.[3] In European Union, nearly 200,000 cases have been reported in 2009.[4]

The *Campylobacter* organism seems to be adapted to birds which they carry without being or showing signs of illness. It has been reported that most people becoming ill with *Campylobacter* manifest a variety of clinical signs including potentially bloody diarrhea, vomiting, nausea, abdominal pain, and fever within few days after exposure to the organism and typically signs lasts for few days.[5] Small percentage of infected people do not express clinical signs however *Campylobacter* can potentially cause more serious and life threatening clinical signs in people with compromised immune system.[6] It has been reported that 50-70% cases of human *Campylobacter* is attributed to consuming contaminated poultry products. This reflects the need for lowering contamination associated with *Campylobacter* thus decreasing occurrence which is considered essential step in lowering incidence rate of human infection. In many countries, culled layer chicken is consumed as part of human diet.[7] In Jordan, layer chickens are sold as spent hens for human consumption at the end of their laying period. There have been a considerable research body that documented and characterized the prevalence and risk factors associated with *Campylobacter* in broiler chicken industry. A local study in Jordan was conducted on samples obtained from broiler birds at the slaughter house indicating a 40% prevalence rate of *Campylobacter*.[8] However, there is limited research efforts conducted to estimate the occurrence rate and identify risk factors associated with *Campylobacter* in layer industry. Therefore, the broad goal of the research project reported here is to determine prevalence of *Campylobacter,* source of infection and risk factors associated with *Campylobacter* infection in layer flocks in Northern Jordan.

Materials and Methods

Sample collection and analysis

Cloaca, litter, water, and feed samples were collected from 35 operating layer farms represented by 43 flocks and a total of 478,600 birds located in Northern Jordan.[9] Each farm/house was visited once, where five cloacal swabs representing 1000 birds were collected (giving a total of 2395 cloacal samples). Sterile swabs were inserted into the cloaca and rotated gently before being pulled out. A total of 500 gm of litter or feed were randomly collected from five different locations from each house/farm and ten different locations water sources (drinkers) were randomly selected to collect a total of pooled 2 litters of water from each house/farm. All cloacal, litter, water, feed samples were harvested utilizing sterile swabs, spoons, containers and gloves then transported to laboratory in an ice box and analyzed within few hours from collection.

Water samples collected from the main water tanks and water drinkers from each farm/house was also analysed to determine

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the level of total chlorine, free residual chlorine and water acidity (water pH). Total and free residual chlorine was measured using DPD (N, N-diethyl-p-phenylene-diamine) method and results were expressed with range of 0 to 3.5 parts per million (ppm) or equivalent to (0-3.5 mg/L). Water acidity was measured electrometrically using pH meter (inoLab, Germany).

Isolation identification and confirmation of Campylobacter

The reference bacterial strains used in this study as positive control were *C. jejuni* ATCC 33291 and *C. coli* ATCC 43478 that was obtained from Jordanian Food and Drug Administration.

The method described by ISO 10272-1:2006(E) was followed for isolation of *Campylobacter*.[10] Samples collected from cloaca, litter, feed or filtered water (0.45 mm membrane filter) were separately diluted with 225 mL of enrichment Bolton broth medium (Oxoid, UK) and homogenized using stomacher (Seaward, U.K) for 2 minutes at 2400 rpm. Obtained homogenates were incubated at 37°C for 4-6 hours and then at 41.5°C for 44 h ± 4 h under micro-aerobic conditions using a gas-generating kit Campygen sachets (Oxoid, UK). After enrichment step, inoculums from each source were inoculated into modified charcoal cefoperazone deoxycholate agar (mCCD agar) (Oxoid, UK) and incubated at 41.5°C under micro-aerobic atmosphere and inspected after 44 h ± 4 h. Two colonies, presumed to be *Campylobacter,* were sub-cultured on a non-selective Columbia blood agar (Oxoid, UK) for purification. Confirmation was done by microscopic examination for morphology and motility followed by oxidase, catalase, DRYSPOT latex agglutination, and Hippurate hydrolysis tests.[11]

All oxidase negative colonies do not require further confirmatory tests. The DRYSPOT latex agglutination test (Oxoid, UK) was performed according to the manufacturer's instructions where the test was considered positive when agglutination noticed within 3 minutes. Hippurate hydrolysis test was done according to standard protocol of the manufacturer's instructions. Hippurate hydrolysis test was considered positive if a dark violet color formed in the testing tubes. The *Campylobacter jejuni* hydrolyzes Hippurate and gives positive results.

PCR molecular typing

DNA extraction and PCR technique was performed as described by Nayak *et al.* (2005) for the amplification of 160bp DNA fragment of the oxidoreductase subunit in the *Campylobacter* genome.[12] The pair of primers used for *C. jejuni*: F 5'- CAA ATA AAG TTA GAG GTA GAA TGT-3'and R 5'- GGA TAA GCA CTA GCT AGC TGA T-3' and for *C. coli:* F 5'- ATG AAA AAA TAT TTA GTT TTT GCA-3' and R 5'- ATT TTA TTA TTT GTA GCA GCG-3' (Alpha DNA, Montreal, Canada) were used to amplify DNA fragment that corresponds to the region of oxidoreductase subunit.

Study area and data collection

A questioner was purposely designed using close end questions. The questionnaires were filled by state veterinarians during a field visit. The questions gathered information to reflect farms environmental current situation and preventive practices in use that might increase or decrease the risk of infections.

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS, version 19.0, SPSS Inc., Chicago, IL, USA). Association between isolation of *Campylobacter* and potential risk factors were initially screened in a univariable analysis using Chi-square test. Only variables with no Collinearity ($r<0.60$) were considered for the univariable analysis. Collinearity was evaluated using non-parametric spearman rank correlation test. Only variable with significant association with *Campylobacter* were considered for the final multivariable logistic regression model. Variables were forced into the multiple regression models using *Enter* method. The Hosmer-Lemeshow test was used to evaluate the goodness-of-fit for the developed logistic regression model. The independent student t-test was used to test for differences between negative and positive farms in regard to quantitative variables listed in Table 1. Statistical significance was set at ($P\leq0.05$).

Results

A total of 535, 13, 7 and 0 isolates were recovered from cloacal swabs, water, litter and feed samples respectively. The number of samples that revealed presumptively identified *Campylobacter* species using

Table 1. Quantitative statistics for *Campylobacter* evaluated risk factors.

Variable	Isolation status	Min	Mean	25th percentile	50th percentile	75th percentile	Max
Age (weeks)	Negative	32	52.00	40.00	52.00	64.00	76.00
	Positive	36	60.00	52.00	64.00	64.00	80.00
Number of birds per farm	Negative	5000	16414	6000	17500	20000	48000
	Positive	3000	11847	6000	11000	16000	20000
Distance between farms (m)	Negative	1000	2200	1750	2000	3000	3300
	Positive	30	380	90	500	500	700
Stocking density (birds/m2)	Negative	5	6.04	5.38	6	7	7
	Positive	9	10.33	10	10	11	12
Water pH (drinkers)	Negative	7.14	7.51	7.30	7.60	7.70	7.80
	Positive	7.28	7.38	7.30	7.40	7.40	7.65
Total chlorine in main water tanks	Negative	0.20	0.28	0.28	0.25	0.37	0.40
	Positive	0.20	0.27	0.21	0.26	0.34	0.40
Residual chlorine main water tanks	Negative	0.00	0.11	0.04	0.10	0.20	0.20
	Positive	0.00	0.09	0.00	0.07	0.20	0.20
Total chlorine in drinkers	Negative	0.00	0.78	0.08	0.05	0.16	0.18
	Positive	0.01	0.08	0.03	0.06	0.15	0.20
Residual chlorine in drinkers	Negative	0.00	0.06	0.00	0.01	0.06	0.07
	Positive	0.00	0.02	0.00	0.01	0.06	0.09

Table 2. Number of PCR confirmed *Campylobacter* jejuni and prevalence rate of positive farms.

Sample source	No. of tested samples	Number of PCR confirmed isolates	Number and prevalence rate among farms [n=35], (%)
Cloaca	2395	535	14 (40)
Litter	43	7	7 (20)
Water	43	13	13 (37)
Feed	43	0	0 (0)
Total	2524	555	14 (40)

mCCD agar was 978 positive out of the 2524 tested samples. All of the 555 Agglutination positive isolates were positive for both Hippurate hydrolysis test and Catalase test and were also confirmed molecularly using PCR technique as *C. jejuni* (Table 2). Isolation sources of *Campylobacter* from positive farms are presented in Table 3.

Univariate binary regression analysis showed no significant association between the evaluated risk factors and the isolation of *Campylobacter.* Also, multiple regression analysis showed no significant association between the isolation of *Campylobacter* and the evaluated risk factors collectively probably reflecting the small sample size of the study farms. Data are presented in Table 4.

The independent student t-test showed only significant difference between negative and positive cloacal isolates in regard to stocking density, height of the fence, distance between farms and water pH. Summary of data are presented in Tables 1 and 4.

The level of total chlorine in the main water tanks and water troughs of the studied farms ranged from (0.2-0.4 ppm) and (0.0-0.2 ppm), while the free residual chlorine ranges were (0-0.2 ppm) and (0-0.09 ppm) respectively (Table 1).

The 160 bp product amplified by the primer sets targeting the gene segment on the oxidoreductase encoding gene of all identified *C. jejuni* isolates were detected in all the 555 isolates by conventional PCR.

Our questionnaire reviled 19 *Campylobacter* positive farms out of the 30 that have no implementation of any hygienic preventive measures. These 30 farms are designated as farms with (Low level of biosecurity). Two *Campylobacter* positive farms out of the 5 that implement some kind of preventive measures were designated as farms with (Medium level of biosecurity). None of the studies farms have (High level of biosecurity).

Table 3. Sample source and percentages of *Campylobacter* positive farms (n=14).

Sample source	Number and (%) positive farms
Water + cloaca + litter	2 (14)
Cloaca + water	3 (21)
Cloaca + litter	5 (36)
Cloaca only	4 (29)
Water only	8 (57)

Discussion and Conclusions

The prevalence rate of *Campylobacter jejuni* isolated from the tested farms in cloaca, water, litter and feed was 14%, 13%, 7% and 0% respectively. The (40%) flock-level prevalence of *C. jejuni* (cloacal swabs) in the tested layer chicken farms is within the suggested prevalence ranges of 2-100% in both developed and developing countries.[13] It is of no difference from other rates detected in broiler chicken from France (42.7%), Denmark (42.5%), Germany (41%), Japan (45%), Italy (80%) , and Jordan (40%).[8,13] In this study *C. jejuni* was isolated either from one or different sources within the same farm. The most highly contaminated source of *C. jejuni* was drinking water. Newell *et al*. (2011) suggesting the horizontal introduction of *Campylobacter* from the environment in a laying hen flock, which may suggest a link between chicken colonization and water or litter contamination.[14]

In other study of *C. jejuni* rout of transmission and possible sources of infection to broilers, it was concluded that water supply was the predominant source of *C. jejuni* infection on the farm.[15]

Positive litter samples were always associated with positive birds. None of the feed samples revealed the presence of *Campylobacter* and this might be expected because of the dryness of the feed that does not encourage the survival of highly sensitive *Campylobacter* species.[16-18]

Epidemiological studies characterizing occurrence and risk factors associated with broiler flocks have been widely conducted in many locations. Several risk factors have been reportedly considered significant in the occurrence of *Campylobacter* in broiler flocks including practicing proper hygienic measures, presence or movement of animals or rodents on the farms or in close proximity, age, and size of the flock.[19-25] However, epidemiological studies characterizing occurrence and risk factors associated with *Campylobacter* in layer farms is not fully investigated in many parts of the world. Findings presented here highlight a set of substantial risk factors that could potentially be associated with occurrence of *Campylobacter* in layer farms.

Univariable or multivariable regression analysis of the evaluated risk factors indicated that there was no statistically significant association between the evaluated risk factors and isolation of *Campylobacter* which could be mostly attributed to the relatively small number of farms tested. However, analysis of the frequency information and data presented in Tables 1 and 4 indicate some risk factors that are associated with substantially higher prevalence of *Campylobacter* infection in layer farms. This include birds stocking density (>6 birds/m^2), short distance between farms (<1000 m), lack of hand washing before entering the farms, presence of litter piled piles inside the farm, presence of rats, mice, pigeons and sparrows. Furthermore, the mean socking bird density in positive farms was significantly higher (10.33 birds/m^2) than negative farms (6.04 birds/m^2). Also, the mean distance between positive farms were significantly shorter (380 m) when compared with negative farms (2200 m). In here, distance was associated with a decreased risk of *Campylobacter* infection which might highlight the role of long dis-

tance for rodents and resident birds. In this study, 60% of the positive farms had higher bird stocking density and 60% of positive farms were within short distance to each other. Also, *Campylobacter* infection was higher on farms that had multiple houses.

Previous epidemiological studies have identified risk factors associated with the prevalence of *Campylobacter* in broiler farms including higher age of chicken, movement or presence of animals around farms.[14-30] In here, 5% of the positive farms had rats or mice while 55% of the negative farms did have neither rats nor mice. Also, 40% of the negative farms did not show any pigeons or sparrows. It has been reported that wild birds are potential contaminants to farms or the surrounding soil.[31] It appears that good hygiene practices by farmers such as changing disinfectant at gate or house door, decontamination of vehicles entering farms, presence of high fence (1.8 m) and secure gait, restricted access of non-essential visitors and wearing protective boots seemed to be associated significantly with lower prevalence rate of *Campylobacter* infection. These findings are in agreement with other previous studies.[14,30] As expected *Campylobacter* was isolated from the litter samples which might also act as a potential source of contamination of chicken houses.[28,29] The potential source of litter contamination is mainly from the intestinal contents and it is expected to survive under its wet condition.

The value of a clean water source is paramount and should be monitored in chicken industry. Chlorine is the most commonly used disinfectant in water treatment of broiler and layer chicken industry. Chlorinating drinking water is helpful in reducing the risk of *Campylobacter* colonization.[26] The recommended level of residual chlorine concentration in poultry farms is from 2-5 ppm within a pH range of 6-8.[27] The residual chlorine values recorded in farms under study were less than 2 ppm which is considered not inhibitory for the growth of *Campylobacter* in chicken drinking water.[27] Chlorine acts predominantly as a sanitizer when the pH of the water is neutral or acidic. In here, the mean values of the pH of drinkers of positive and negative farms are 7.38 and 7.51 respectively. Data are summarized in Table 1.

One limitation of this study is the limited number of farms studied. The study farms were the only available farms in the region during the study period. In Jordan, there are no integrated layer companies and their production is mostly dependent on individual layer farmers.

The significance of the study reported here is being new efforts conducted in layer farms to determine the prevalence rate and characterize isolation of *Campylobacter* and highlight important risk factors for their occurrence in layer farms.

The authors believe that the finding presented here is still relevant despite the lack of finding significant association between the evaluated risk factors and the isolation of *Campylobacter* in layer farms. It is recommended to improve hygienic practices at layer farms and to establish national guidelines and biosecurity standards to decrease prevalence rate of *Campylobacter* in layer farms which would positively impact the poultry industry and lower infection rate of human *Campylobacter*.

Table 4. Variables included into the final multivariable logistic regression analysis of their association with *Campylobacter* isolation (data presented as proportions).

Variable or risk factors	Category	*Campylobacter* isolation from the farm	
		Positive	Negative
Stocking density (Birds/m2)	≤6	0	9
	>6	21	5
Distance between farms (m)	<1000	21	0
	≥1000	0	14
Decontamination of vehicles outside the farm	Yes	1	7
	No	20	7
Fence (1.8 meter height) with secure gait	Yes	0	6
	No	21	8
Hand-washing before entering farm/house	Yes	3	3
	No	18	3
Changing disinfectant in the pool	Yes	8	14
	No	13	0
Disinfection at house door	Yes	8	14
	No	13	0
Restricted access of vehicle and non-essential visitors	Yes	1	14
	No	20	0
Presence of disinfectant in the pool at farm gait	Yes	1	7
	No	20	7
Piles of litter inside the farm	Yes	10	0
	No	11	14
Presence of rodents (mice and rats)	Yes	2	4
	No	19	10
Presence of sparrows or pigeons	Yes	13	0
	No	8	14
Biweekly changing the pool disinfectant	Yes	1	7
	No	20	7
Protective boots	Yes	3	14
	No	18	0
Sanitation/room facility	Yes	7	11
	No	14	3

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