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Effect of Different Hygienic levels on *Salmonella* and Antimicrobial Resistance in Layer Cages System

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Abstract: More and more concerns about the regular use of antimicrobials in poultry production, which led to the emergence of antibiotic resistant bacteria. From human health prospective, antimicrobial resistance constitutes a real threat to public health. Therefore, this study was carried out to compare between low and high hygienic layer cages system practices to decrease the risk of *salmonella* and antibiotic resistant bacteria. The obtained results showed that the highest incidence of aerobic bacteria and *salmonella* was observed in low hygienic layer cages with excessive usage of antibiotic. In contrast, in high hygienic layer cages system with prudent antibiotics, the lowest counts of aerobics and incidence *salmonella* was obtained. The *Salmonella* serotypes were 41.04% of *S.typhimurium* followed by *Salmonella Enteritidis* was 30.35%. *Salmonella* species showed the highest sensitivity against more than 50% of tested antibiotics. The present study demonstrated the beneficial effect of hygienic system to reduce with the risk of pathogenic bacteria.

Key words: Hygienic level · Hen housing · *Salmonella* · Antimicrobial Resistance

INTRODUCTION

Hygiene is a set of practices performed to maintain health. According to the World Health Organization (WHO), "Hygiene refers to conditions and precautions that help to preserve health and prevent the spread of diseases. Application of hygiene and biosecurity measures in poultry farms, including efficient cleaning and disinfection procedures, control of vermin, change of footwear at the entrance of each layer house and other hygienic practices which reduce introduction of infection from the farm environment to the birds [1]. There are different environmental factors which affecting farm hygiene as feces of previous flocks, Salmonella infection of day-old chicks, temporary workmen, work equipment, feed and feeders, water and drinkers [2].

Enterobacteriaceae as well as aerobic bacterial count in poultry farms can be routinely used as indicators of improper hygienic which can lead to proliferation of pathogens, such as salmonella [3].

Salmonella is the causative agent of the majority of gastrointestinal illnesses. It is responsible for the infecting about 100 million human infectious annually, not only in developing countries but also in developed communities, where salmonellosis is still not vanquished. *Salmonella* could cause severe disease in humans, such as gastroenteritis and typhoid fever [4]. It is a ubiquitous and hardy bacterium that can survive several weeks in a dry environment and several months in water [5].

Antibiotics are used in the poultry industry to enhance growth, productivity of flock and reduce disease. Also, it enhances the health and well-being of poultry by reducing the incidence of disease. Although these uses benefit all involved, unfortunately, there is the perception among many consumers that our food supply contains high concentrations of drug or hormone residues causing significant health concerns or problems [6].

Antimicrobial resistance occurs when bacteria change in response to the use of these medicines. Antimicrobial agents supply the selective pressure to expansion of resistance and encourage the transfer of resistance genes

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among bacteria although; physical characteristics of the microbial community play a major role in gene exchange [7]. Already, many countries have taken decision to decrease the use of antibiotics in food-producing animals. The use of antibiotics for growth promotion banned in the European Union has banned. Prevention of animal disease can achieves by hygiene improving, proper vaccination and changing in animal housing and management without using of antibiotic [8]. The prevention of resistance development to new antibiotic is essential control of antibiotic usage. The present study aimed to evaluate the effect of different housing hygiene levels for laying hens on the prevalence of total bacterial count, *Salmonella* and antimicrobial resistance in *salmonella*.

MATERIAL AND METHODS

Samples: A total number of 210 samples and swabs was collected from two layer farms (n=105 from each farm). The samples were collected from wall, roof, feces, water sources, drinker, feed storage and feeder (n=15 of each from each farm).

Farms: The first farm has open ventilation system and supplied with positive pressure fan. The drinkers and feeders were manual. Water source was underground water without any treatment, there weren't water tanks, the drinkers weren't washed frequently, feeding was manufactured feed and its storage time was 1 week. The manure evacuation was manually every day outside the farm. The disinfection of the farm was carried out by dry cleaning (without remove of organic matter) followed by moist cleaning using commercial chlorine; all steps were carried out in the same day. The terminal disinfection was absent. The farm contained neither foot path nor a fence. The farm wasn't protected from rodent nor wild birds. The enrofloxacin antibiotic was added to water as prophylactic measures against microorganism (water dose adjusted to give 10mg/kg bw/day).

The second farm has closed ventilation system. The drinkers and feeders were automatic. Water source was tap water; there was water tank in each pen which was disinfected with water tubes by virkon S® (potassium per sulfate). The drinkers were washed frequently every, feeding was unmanufactured feed and its storage time was 2 days. The manure evacuation was automatic every day outside the farm. The disinfection of the farm carried out by dry cleaning and removed all organic matter followed by moist cleaning by water and soap. Then, the pens was disinfected by commercial chlorine then by commercial iodine everyone alone followed by spraying by TH4® (quaternary ammonium chloride and glutraldhyde). The farm contained foot path and surrounded by a fence both of them were disinfected with T4® and Th4®; the farm was protected from rodent and wild birds. The erythromycin antibiotic was added to water as prophylactic measures against microorganism by dose (100-1500mg/l) daily.

Samples Preparation: The tubes containing swabs, fecal material, feed and water samples were pre-enriched in saline solution (0.9 % NaCl) in 1:10 ratio (10 gm or mL in 90mL saline solution).Serial dilutions of the sample were prepared up to 10^{-6}.

Preparation of Swabs [9]: A tenfold serial dilution was prepared by mixing of 225ml of sterile buffered peptone water with the collected swap to make (1/10) dilution. In a sterile test tube 1 ml of the dilution was mixed with 9ml of buffered peptone water.

Aerobic Plate Count (APC) Was Determined [10]: One ml from each dilution was transferred aseptically onto duplicate petri dishes and then tryptic soy agar medium was added, to which melted and cooled to 45°C standard plate count agar was added. The plates incubated aerobically at 37 ° C for 48 hr. The aerobic plate counts were estimated per gram on the plate containing (30-300) colonies.

Isolation of Salmonella [11], [12]: From the original dilution 1ml was inoculated into sterile peptone water then was incubated at (37°C) for (18) hours for pre-enriched for *salmonella.* From each pre-enriched culture 1ml was transferred into 9 ml Rappaport-vassiliadis soya peptone (RVS) and incubated at 41.5°C for 20-24hr. From enriched broth culture, a loopful was streaked separately onto Salmonella selective agar plates; Xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar and Brilliant green (BG) agar and incubated at 37°C for 24-48hr. Suspected *Salmonella* colonies showed Blue-green to blue colonies with or without black centers on HE agar, Pink colonies with or without black centers on XLD agar and pink colonies surrounded by brilliant zone on BG agar. One colony from the presumptive salmonella colonies were sub-cultured on MacConkey agar. The typical colonies appear transparent and colorless, sometimes with dark center. One separated presumptive salmonella colony was transferred to nutrient broth for further identification.

Table 1: Antimicrobial discs, concentration and interpretation of their action on the isolated Salmonella species.

Antimicrobial agent	Sensitivity disc content (ug)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Nalidixic acid (NA)	30	13 or less	14-18	19 or more
Oxytetracycline (T)	30	14 or less	15-18	19 or more
Ampicillin/Sulbactam (AS)	20	13 or less	14-17	18 or more
Gentamicin (G)	10	12 or less	13-14	15 or more
Kanamycin (K)	30	13 or less	14-17	18 or more
Enrofloxacin (EN)	5	11 or less	12	13 or more
Amikacin (AK)	30	12 or less	13-15	16 or more
Streptomycin (S)	10	11 or less	12-14	15 or more
Cefotaxim (CF)	30	17 or less	18-22	23 or more
Neomycin (N)	30	12 or less	13-16	17 or more
Erythromycin (E)	15	13 or less	14-22	23 or more
Ciprofloxacin (CP)	5	15 or less	15-19	20 or more
Cephalothin (CN)	30	14 or less	15-17	18 or more
Sulphamethoxazol (SXT)	25	10 or less	11-15	16 or more

Biochemical Identification [13, 14]: The following biochemical tests were carried out on purified colonies for their confirmation as *Salmonella* and ruling out other *Enterobacteriaceae*. Indole test (Negative), H_2S production (positive), citrate test (positive), methyl-red test (-positive), Voges-Proskauer test (negative), lysine decarboxylase test (positive), ornithine decarboxylase test (positive), urease test (negative), sugar fermentation (sucrose, lactose and salicin (negative), xylose (positive) Gelatin liquefaction (negative) and motility test (predominately motile).

Serological Identification of Salmonellae: Serological identification of *Salmonellae* was carried out according to Kauffman-White scheme [15] for the determination of Somatic (O) and flagellar proteins (H) antigens using *Salmonella* antisera (DENKA SEIKEN Co., Japan). The identification of somatic (O) antigen was carried by slide agglutination test by using *salmonella* Polyvalent "O" antisera and determination of Flagellar (H) antigens was carried out by using Polyvalent H antisera for both phase 1 and phase 2 in tube agglutination test.

Antibiotic Resistance of Salmonella Species: The disk diffusion method [16] was used to test the sensitivity of Salmonella species using 14 antibiotics. Therefore the inhibition zones were measured and scored as sensitive, intermediate and resistant according to according to the guidelines stipulated by National Committee for Clinical Laboratory Standards [17]. Accordingly, the antimicrobial discs and their concentrations as well as the diameters of the zones of inhibition for the tested strains are demonstrated in Table 1. The tested strains were evaluated as susceptible, intermediate and resistant. Multiple Antibiotic Resistance (MAR) index for each strain was determined according to the formula [18] as follow:

MAR index = No. of resistance (Isolates classified as intermediate were considered sensitive for MAR index) / Total No. of tested antibiotics.

Statistical Analysis: The statistical analyses were done by univerate analysis and one way ANOVA using SPSS program version 20. P value < 0.05 was assumed for statistical significance.

RESULTS

Aerobic Plate Counts Bacteria: The average total counts of aerobic bacteria from the collected samples in low hygiene and high hygiene are shown in Table (2) and illustrated by Fig (1). Data emphasized that the significant highest values of aerobes were observed in low hygiene compared to high hygiene. The extreme average level of aerobic bacteria was found in the fecal swab (7.42± 0.08 and 6.02 ± 0.08 log (cfu/g) followed by wall swaps (6.52±0.11 and 5.23± 0.08 log (cfu/g) for low and high hygiene farms, respectively. While, water source and preserved food gave the lowest average level of aerobes were (5.26±0.09 & 5.31±0.09 log (cfu/g) and (4.16±0.08 & 3.91±0.09 log (cfu/g) for low and high hygienic housing system, respectively.

Salmonellae Incidence and Identification: The suspected colonies of *salmonella* were 100% in fecal swap, 66.67% in wall and drinking water, 46.67% in feeder food, 33.33% in roof and 26.66% in water source and preserved food. On the other hand, the suspected colonies of *salmonella* were detected only in 6.66% of fecal swaps (Table 3).

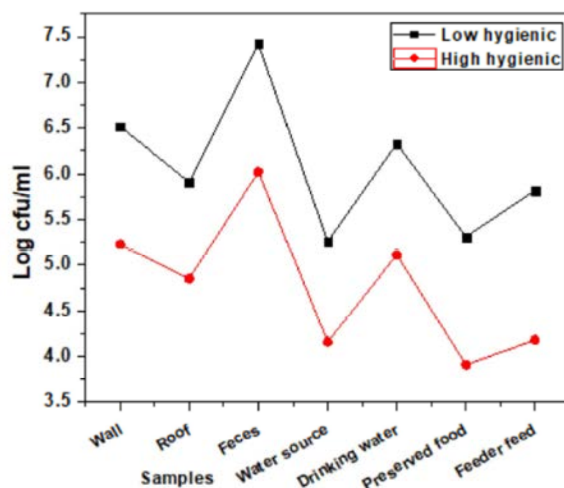

Fig. 1: APC (log 10 cfu/g) at different hygienic layer cages housing system.

Table 2: Aerobic plate count bacteria (log 10 cfu/g) in different hygienic measure of layer cages housing system.

Samples	Low hygiene	High hygiene
Wall	6.52 ± 0.11 [a]	5.23 ± 0.08 [b]
Roof	5.91 ± 0.05 [a]	4.85 ± 0.06 [b]
Feces	7.42 ± 0.08 [a]	6.02 ± 0.08 [b]
Water source	5.26 ± 0.09 [a]	4.16 ± 0.08 [b]
Drinking water	6.33 ± 0.09 [a]	5.11 ± 0.07 [b]
Preserved food	5.31 ± 0.09 [a]	3.91 ± 0.09 [b]
Feeder feed	5.82 ± 0.06 [a]	4.18 ± 0.09 [b]

The means with different superscripts in the same column indicate significant difference; the significant difference is at the <0.05 level.

Table 3: The incidence of *salmonella* in different hygienic measure of layer cages housing system.

	Low hygiene		High hygiene		Total	
Samples	No of +ve	%	No of +ve	%	No of +ve	%
Wall	10/15	66.67	0/15	0	10/30	33.33
Roof	5/15	33.33	0/15	0	5/30	16.67
Feces	15/15	100	1/15	6.66	16/30	53.33
Water source	4/15	26.66	0/15	0	4/30	13.33
Drinking water	10/15	66.67	0/15	0	10/30	33.33
Preserved food	4/15	26.66	0/15	0	4/30	13.33
Feeder feed	7/15	46.67	0/15	0	7/30	23.33
Total	55/105	52.3	1/105	0.83	56/210	26.67

Table 4: Salmonella serotypes incidence and serological identification in different hygienic measure of layer cages housing system

	Type of farms				Antigenic structure	
Salmonella serotypes	Low hygiene measure	High hygiene measure	No. of serotypes	%	O	H
S.typhimurium	+	-	23	41.07	6,7	r : 1,5
S.enteritidis	+	+	17	30.35	8,20	i : Z6
S. kentuckey	+	-	9	16.07	1,4,5,12	i : 1,2
S.molade	+	-	4	7.1	8,20	Z10 : Z6
S.infantis	+	-	3	5.35	1,9,12	g,m : -
Total	55	1	56	26.67	-	-

The *salmonella* serotypes were 41.07%, 30.35%, 16.07%, 7.1% and 5.35% of *S.typhimurium, S.enteritidis, S. kentuckey, S.molade, S.infantis,* respectively. All salmonella serotypes were isolated from low hygienic layer cages farm, in contrast only one strain was isolated from high hygienic layer cages farm was serotypes as *S.enteritidis* (Table 4). For identify the *salmonella* species (0 antigen and the H antigen identification) was used. The identified strains and the identification of antigens 0 and H correctly were observed in (Table 4).

Table 5: Antibiotic susceptibility of isolated Salmonella species

	Sensitive (S)		Intermediate (I)		Resistant (R)	
Antimicrobial agent	NO	%	NO	%	NO	%
Streptomycin	-	-	-	-	21	100
Erythromycin	-	-	1	4.8	20	95.2
Nalidixic acid	1	4.8	3	14.3	17	80.9
Sulphamethoxazol	2	9.5	5	23.8	14	66.7
Cefotaxim	4	19.0	3	14.3	14	66.7
Oxytetracycline	5	23.8	3	14.3	13	61.9
Cephalothin	7	33.3	4	19.0	10	47.6
Amikacin	10	47.6	2	9.5	9	42.9
Neomycin	12	57.1	1	4.8	8	38.1
Enrofloxacin	13	61.9	2	9.5	6	28.6
Kanamycin	14	66.7	2	9.5	5	23.8
Ampicillin/Sulbactam	15	71.4	3	14.3	3	14.3
Ciprofloxacin	18	85.7	1	4.8	2	9.5
Gentamicin	19	90.4	1	4.8	1	4.8

Table 6: Antimicrobial resistance profile of isolated Salmonella species

Strains	Antimicrobial resistance profile	MAR index
S. *Typhimurium*	S, E, NA, SXT, CF, T, CN, AK, N, EN, K, AS, CP, G	1
S. *Enteritidis*	S, E, NA, SXT, CF, T, CN, AK, N, EN, K, AS, CP	0.928
S. *Kentucky*	S, E, NA, SXT, CF, T, CN, AK, N, EN, K, AS	0.857
S. *Infantis*	S, E, NA, SXT, CF, T, CN, AK, N, EN, K	0.786
S. *Molade*	S, E, NA, SXT, CF, T	0.428

Antibiotic Susceptibility of Salmonella Species: The antimicrobial sensitivity patterns of *Salmonella* species were shown in (Table 5; 6). *Salmonella* species showed the highest sensitivity to gentamicin and ciprofloxacin were 90.4% and 85.7%, respectively where as the complete resistant seen was to streptomycin was 100% followed by erythromycin (95.2). *Salmonella* species showed sensitivity to ampicillin/Sulbactam, kanamycin, enrofloxacin and neomycin above 50%, intermediate (14.3- 4.8 %) and resistant ranged from 14.3 to 38.1 %. In the contrary, *Salmonella* species showed resistant to nalidixic acid, sulphamethoxazol, cefotaxim, oxytetracycline, cephalothin and amikacin in rang 80.9 to 42.9 % and intermediate ranged from 14.3 to 9.5 %.

DISCUSSION

For the time being, there are three aspect are strongly linked to each other in food production, food safety, protection of environment and animal welfare. The consumers who have more and more concerns. The implementing regulations or new standards to improve one aspect may negatively influence other aspects, creating potential conflicts between regulatory aims [19]. Farm hygiene and biosecurity level are critical points in poultry industry, any defects in farm hygiene can cause bacterial contamination, diseases introduction, economical losses and serious public health hazard.

Our results showed that the total aerobic plate counts bacteria were high in fecal swabs in both farms compared to other treatments. Moreover, the number of microorganisms in the samples is considered as an indication of its hygienic or not. These results agreement with those obtained by Witkowska and Sowiñska [20] and Kostadinova *et al.* [21] who revealed that aerial contamination in the range of 4.7-8.3 $\log_{10}$ cfu/g in laying hen houses.

In the worldwide poultry industry, *Salmonella* remain very challenging diseases due to the inefficiency of implementing, integrating eradication and control programs, which results in very high economic losses to the poultry industry [22]. The *salmonella* was isolated from wall, roof, fecal swap, water and feed in different layer farms hygiene. The incidence of salmonella was the highest in the fecal swaps (100%), followed by (66.67%) in wall and water that collected from low hygienic layer cages house. In contrast, the salmonella was detected only in 6.60% of fecal swap that collected from the high hygienic layer cages house. The layer housed in high hygienic farm was had a lower infection of *salmonella* in comparison to layer housed in low hygienic farm. The main source of *salmonella* contamination in layer cages farm are from surround environment, dust, insects, free living animals and rodent [23].

The highest prevailed *Salmonella* serotypes were *S.typhimurium* at prevalence (41.07%), followed by

S.enteritidis (30.35%), *S. kentuckey* (16.07%), *S.molade* (7.1%) and *S.infantis* (5.35%). *S.typhimurium* followed by *S.enteritidis* were the dominant bacterial species in the intestinal microbiota in laying hens housed in cage systems and there are the most commonly isolated serotype in human cases of salmonellosis [24, 25]. An important zoonotic pathogen typically associated with eggs and egg products is *Salmonella Enteritidis* [26, 27]. Association between the level of hygiene, biosecurity and the occurrence of *Salmonella* was strong. It has been indicated that proper biosecurity measures should be in place to lower the occurrence of *Salmonella* [22].

It was noted that poultry farms rely heavily on the use of antibiotics to control diseases and that all farms used one or more antibiotics to promote therapeutic, preventive and to a lesser extent to promote growth. In this study, *salmonella* species showed resistant to both streptomycin and erythromycin; it's may be due to the continuous use of them led to *Salmonella* resistance [28, 29]. The present results showed that using ciprofloxacin and gentamicin had highest effect against *Salmonella* species but these antibiotics very expensive. These antibiotics are expensive and are the drug of choice in the treatment of invasive enteric infections both in animals and humans [30]. Although high hygienic layer farm used salmonella-resistant antibiotics (erythromycin), it had a very low incidence of *salmonella* compared to a low-grade healthy farm that used a strong antibiotic (enrofloxacin) and had a high incidence of salmonella. This means that a high level of hygienic measure will restrict the using of antibiotics in the poultry farm, will providing a high-quality and safe food and food products.

CONCLUSION

Finally, the high hygienic and biosecurity levels of layer cages housing farms will reduce the use of antibiotic in water or food as prophylactic measure. Furthermore, we will avoid the drug residues in egg, bird meat. In addition to, we will prevent the antimicrobial resistance and will reduce the cost of antibiotic usage.

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