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Article

Antibiotic Resistance in Layers Production Chain Outlook in a One Health Approach

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Abstract: This study was funded by the National Agency for Scientific Research and Innovation (NASRI) in Albania, under the contracted project "Antimicrobial resistance in layers production chain: Risks and "One Health" approach implication". Avian colibacillosis and salmonellosis are the major bacterial diseases in the poultry industry world-wide. The purpose of this study was to determine the prevalence of antimicrobial resistance patterns in *Escherichia coli* and *Salmonella* spp. isolated from healthy layers breaded in establishments located in geographical proximity during different stages and ages of their production cycle. Through a cross-sectional study and boot swabs were collected from 5 layers establishments, feed and egg storage units, in each compounding barn of the farm, respectively. In recent decades, across with the development of the intensive poultry industry, antimicrobial resistance (AMR) has become a global veterinary public health concern. Several studies have assessed AMR in commensal and pathogenic bacteria from different poultry species and breeding in Albania, there is a lack of studies that compared AMR in commensal *E. coli* and *Salmonella spp*. isolated from laying hens' establishments for table eggs production. A total of 120 of swab boots samples, were collected by three rounds of sampling (every four weeks) from each unit of 5 laying hens' establishments and their respective feed and egg storage units, all located in one district, in Central Albania. Clustering analyses and the age of group/unit indicator were applied to identify patterns and differences in AMR. Isolated strains were tested for susceptibility to 14 antibiotics using disk diffusion method. 14 different antibiotics were tested for expanding the antibiotic resistance profiles among the isolated *E. coli*. The rates of resistance toward the antibiotic panel variated from 20% to absolute resistance (100%). Overall, 83/83 (100%) *E. coli* isolates were resistant to at least two antibiotics, Metronidazole and Tylosine. A total of 49/83 (59,03%) of the *E. coli* isolates were Multi Drug Resistant as they were simultaneously resistant to three or more antibiotics from different classes. The most MDR pattern was seen with resistance to T 30, TE 30, TY, DO30, STX 25 and MET, while the least frequent pattern was seen with resistance to ENR5, CIP5, NX10 and COT25.

Keywords: layers production; antibiotic resistance; Albania; colibacillosis; salmonellosis; One Health

1. Introduction

Central region of Albania is nationally recognized as one of the most intensive and quantitative geographical of the breeding and production of laying hens. Localization in narrow geographical areas and of high-density laying hens' establishments during the production cycle, stimulation by medicated feed and or treatments for various infectious diseases, contributes on the risk related to the contamination of environment within and across the productive area of flocks. Moreover, many establishments of large capacities consist in different breeding units, according to flock age. Having more than one production unit within the farm often results in their compromising optimal biosecurity conditions. High density of poultry is another critical point of health control, and also the flock - welfare. The quality of the poultry housing environment plays a vital role in disease expression. Crowded and poorly ventilated conditions can exacerbate the spread of *E. coli*, leading to more severe clinical signs [15]. Ventilation as added to the density of flock may create optimal conditions for the quick development and transmission of pathogenic microorganisms within the poultry flock in a production, between units of production, as well as between establishments that are in geographical proximity.

The age factor for laying hens plays a fundamental role in the development of various infectious diseases, treatments through the production cycle may increase the possibility of the development of antimicrobial resistance in commensal and pathogenic bacteria, present at the flock level. The poultry flock health is affected by their age, because the immunity varies according to the physiological stage of the bird, where young chicks have an immature immune system, making them more susceptible to *E. coli* infections. Human actions to promote production, including improper use of antimicrobial agents in livestock production, have hastened the emergence and diffusion of antimicrobial resistant bacteria [4]. Likewise, for the control of infections in poultry, treatments with active antimicrobial substances are used continuously, causing that with the advancement of the age of the birds, the exposure and accumulation of antibiotic substances increases and, consequently, the probability of the development of antibiotic-resistant bacterial strains increases.

Colibacillosis is a widespread bacterial infection affecting poultry globally [3]. Its prevalence can vary geographically, with certain regions experiencing higher incidence rates due to differences in management practices, climate, and biosecurity measures [15]. On the other hand, poultry serves as the main reservoir for various non-typhoidal *Salmonella* (NTS) serotypes among food-producing animals [7]. In Albania *Salmonella* spp. and Escherichia coli have been isolated from poultry and table eggs and their contents and is previously reported that Salmonella, *E. coli*, can cause diseases in different poultry species and breeding, contamination of the poultry chain, outbreaks in table eggs and subsequent infections in consumers [2, 11, 13 - 14].

Antimicrobial resistance (AMR) is a global health threat that has limited the options for the treatment of bacterial infections in animals and humans [6]. The widespread use of antimicrobials in food animal production, including poultry production, raises a significant global public health concern that is often associated with the emergence of resistance against antimicrobials that are commonly used in those animals [7]. Albania practices a cautious regulatory approach concerning the use of critically important antimicrobials in food-producing animals. In a global context, antimicrobials such as antibiotics are widely used, at non- therapeutic dosages, as growth promoters, and prophylaxis in chicken feeds, and water, which sometimes last for long period, there- by predisposing to emergence of AMR strains of microorganisms [16].

2. Materials and Methods

This study was conducted in five laying hens' establishments, containing from one barn to 5 barns each. All the 15 units/barns have been established for poultry production from the year 1995 to 2010 and were in proximity in one district of Central Albania, operating as individual farms. In this study, one barn is defined as a group of birds that are housed in separated unit, but they share the same airspace of the trademark of the laying hens' establishment. The ventilation of the units was

regulated independently. The average of poultry flocks variated from 18,500 to 320,000 caps per establishment (Table 1).

Table 1. General data of the 5 sampled laying hens' establishments.

Laying Hen establishments	Number of caps	Number of poultry units/barns	Employee	Start	Biosecurity plan	Feed supply	Refill of barns/units	Origin of pullets
No. 1	145,000	3	80	2010	yes	Own production	Day old chicks	Greece Bulgaria
No. 2	70,000	3	35	2002	yes	Own production	all in, all 'out	Greece Bulgaria
No. 3	18,500	1	11	1995	yes	Own production	Day old chicks /	Albania
No. 4	320,000	5	60	2000	yes	Own production	all in, all 'out	Austria Poland
No. 5	150,000	3	35	2004	yes	Own production	105 days old/ all in, all 'out	Netherland Hungary
Total	703,500							

Floor in and Around barns

A total of 120 boot-swab samples, were monthly collected 3 times from five laying hens' establishments, their feed storage and egg collection units, located in one district in central Albania. All samples were aseptically collected and then packaged in sterile polythene zip bags and carried to the laboratory at the Faculty of Veterinary Medicine, Agricultural university of Tirana, in aseptic conditions in a cold box within two hours from the time of obtain. Duplicate samples were obtained whenever possible. All samples were analyzed within 2-4 hours after their arrival to the laboratory. Boot-swabs (moistened in 0.8% sterile saline solution) were worn over the boots, which had been disinfected previously. The samples were taken by walking on the floor inside the layers' barns, feed and egg storage units. Each pair of boot-swabs covered about 50% of the floor. Swabs from one poultry house were pooled into one sample in a sterile plastic bag.

Antimicrobial Susceptibility Testing: Susceptibility tests were performed using the Kirby-Bauer method on Mueller-Hinton agar in accordance with Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines [8] and using 14 antibacterial agents: Florfenicol (FFC 30 mcg), Ciprofloxacin (CIP 5 mcg), Co-trimoxazole (COT 25 mcg), Tetracycline (TE 30mcg), Doxycycline (30 mcg), Metronidazole (MET 10 mcg), Enrofloxacin (ENR 5 mcg), Tylosine (TY 30 mcg), Trimethoprim sulphametaxosole (STX 25 mcg) Amoxycillin clavulanic acid (AMC 30 mcg), Oxytetracycline (T 30 mcg), Norfloxacin (NX 10 mcg), Neomycin (N 10 mcg), and Novobiocin (30 mcg).

3. Results and Discussion

Cross sectional data from the 5 sampled laying hens' establishments revealed important information on the presence of *Escherichia coli* and *Salmonella* spp. according to the sampled unit. In Table 2 is presented age relation of the sampled animals, in each breeding barn, on the three rounds of farm sampling. Similarities were found among the different layers' barns of the same laying hens' establishment.

Table 2. Layers age relation to bacterial isolation, in different layers barns.

Poultry Establishment	Age of Layers First sampling	Positive Samples of *E. coli* 30 samples	Positive Samples of *Salmonella spp.* 30 samples	Age of Layers Second sampling	Positive Samples of *E. coli* 30 samples	Positive Samples of *Salmonella spp.* 30 samples	Age of layers Third sampling	Positive Samples of *E. coli* 30 samples	Positive Samples of *Salmonella spp.* 30 samples
No. 1	73 weeks	2	0	77 weeks	2	0	81 weeks	2	0
No. 1	45 weeks	2	0	49 weeks	2	0	53 weeks	1	0
No. 1	24 weeks	1	0	28 weeks	2	0	32 weeks	2	0
No. 2	70 weeks	1	0	74 weeks	1	0	78 weeks	2	0
No. 2	33 weeks	2	0	37 weeks	1	0	41 weeks	2	0
No. 2	21 weeks	2	1	25 weeks	2	0	29 weeks	2	0
No. 3	57 weeks	2	0	61 weeks	1	1	65 weeks	2	0
No. 4	98 weeks	2	0	102 weeks	2	0	106 weeks	2	0
No. 4	46 weeks	2	0	50 weeks	2	0	54 weeks	2	0
No. 4	46 weeks	2	0	50 weeks	2	0	54 weeks	2	0
No. 4	23 weeks	2	1	27 weeks	2	0	31 weeks	1	0
No. 4	22 weeks	2	0	26 weeks	1	0	30 weeks	1	0
No. 5	59 weeks	2	0	63 weeks	1	0	67 weeks	2	0
No. 5	28 weeks	2	0	32 weeks	2	0	36 weeks	2	1
No. 5	28 weeks	2	0	32 weeks	2	0	36 weeks	2	1
Total		28 strains 93.3 %	2 strains 6.67 %		25 strains 83.3 %	1 strain 3.3 %		26 strains 86.6%	2 strains 6.6%

Avian colibacillosis has been noticed to be a major infectious disease in birds of all ages [5]. The results of isolation for *E. coli* confirmed the persistence of these bacteria in the poultry house with a median rate of identification as 87.77 %, in the three rounds of samplings. The risk for colibacillosis increases with increasing infection pressure in the environment. A good housing hygiene and avoiding overcrowding are very important. Other principal risk factors are the duration of exposure, virulence of the strain, breed, and immune status of the bird. Avian *Salmonella* infections are important as both a cause of clinical disease in poultry and as a source of food-borne transmission of disease to humans. Although infection in newly hatched chicks by nasal and cloacal route are also considered as the important route of transmission. Chicks may be infected early by vertical transmission either from an infected ovary, oviduct or from the infected eggs during the passage through the cloacal feces from infected or carrier hens. The birds survive from clinical disease when infected in young stage may show few signs of infection, but they become carriers [1]. We found no relation between the distribution of *E. coli* strains and the layers age as mass bacterial presence was encountered. No treatment of layers was allowed prior and during the three rounds of samplings. Higher prevalence was found in older layers ages, and no relation of prevalence shift was encountered between samplings.

Moderate rates 5.55% of Salmonella spp. were detected during the study. The presence of *Salmonella* spp. was encountered more frequently in the early stages of production, and not detected in the feed and egg storage units.

In this study, avian *E. coli* and *Salmonella* spp. strains from healthy layers, respective establishments' feed and egg storage units were isolated in the same period in laying hens' establishments located in geographical proximity as the isolates were compared. The study results, as presented in Table 3, suggest that the bacterial carriage of these establishments was found similar between group ages and units within the layers farm, with higher bacterial prevalence in the Layers' farms which had more than one breeding barn, indicating the lack of biosecurity measures applied for the separation of the population of each poultry flock.

Table 3. Isolation of *E. coli* and *Salmonella* spp. strains, according sampling site.

Sampling site	**Establishment No. 1 Two Barns**		**Establishment No. 2 Two Barns**		**Establishment No. 3 One Barn**		**Establishment No. 4 Five Barns**		**Establishment No. 5 Three Barns**	
	E. coli	*Salmonella spp.*	*E. coli*	*Salmonella spp.*	*E. coli*	*Salmonella spp.*	*E. coli*	*Salmonella spp.*	*E. coli*	*Salmonella spp.*
Layers' barn	16	1	16	1	5	1	27	1	17	2
Feed storage unit	2	0	3	0	2	0	1	0	3	0
Egg storage unit	3	0	3	0	3	0	2	0	2	0

Environmental monitoring showed that the distribution of bacterial contamination outside the layer's units, in other barns and units through the farm, is a concern that shall be addressed by the correct application of the internal biosecurity. Schulz et al. [9] suggested that the presence of *Salmonella* during a laying-period is affected by the relatively low within- flock prevalence of shedding hens even in a *Salmonella*- contaminated environment. Horizontal transmission of *E. coli.* was confirmed in 13 cases out of 15 samples (86.66%) in egg storage units of each sampled establishment due to mutual collection unit for all the eggs produced in different barns, meanwhile for feed storage units the bacterial isolation rate was 11 strains out of 155 samples (73.33%).

Table 4. Antibiotic resistance rates of 83 *E. coli* strains, %.

Sampling site	TE 30	T 30	ENR 5	CIP 5	ET 5	DO 30	N 10	NV 10	FFC 30	NX 10	AMC 30	STX 25	Y 30	COT 25
Establishment No. 1 16 strains	75	87.5	56.25	37 .5	0	68.75	75	43 .7	25	43.7	37.5	68.7	0	37.5
Feed storage unit No. 1 2 strains	50	50	0	0	00	0	50	50	0	50	33.3	50	0	0
Egg storage unit No. 1 3 strains	33.3	0	0	33.3	0	0	33.3	33.3	0	33.3	0	66.67	10	33.3
Establishment No. 2 16 strains	62.5	68.75	53.5	31.25	100	62.25	50	31.25	43.75	56.25	53.5	62.25	100	25
Feed storage unit No. 2 3 strains	66.67	33.3	33.3	33.3	100	33.3	66.66	0	33.3	33.3	0	33.3	100	0
Egg storage unit No. 2 3 strains	66.67	33.3	0	33.3	100	0	33.3	33.3	0	0	33.3	66.67	100	33.3
Establishment No. 3 5 strains	60	80	20	40	100	60	60	40	20	60	40	80	100	20
Feed storage unit No. 3 2 strains	50	50	0	50	100	50	50	50	50	50	0	50	100	0
Egg storage unit No. 3 3 strains	66.67	33.3	33.3	0	100	66.67	66.67	33.3	0	33.3	33.3	66.67	100	0
Establishment No. 4 27 strains	74	44.44	33.3	29.62	100	70.37	51.85	40.74	37.03	44.44	40.74	70.37	100	33.3
Feed storage unit No. 4 1 strain	0	100	0	50	100	100	100	100	100	0	0	100	100	0
Egg storage unit No. 4 2 strains	50	50	0	50	100	0	50	50	50	0	0	0	100	0
Establishment No. 5 17 strains	70.58	76.47	47.05	29.41	100	64.70	58.82	52.94	58.82	58.82	64.70	35.29	100	29.41
Feed storage unit No. 5 3 strains	66.67	33.3	33.3	33.3	100	33.3	66.67	67.67	33.3	33.3	0	66.67	100	0
Egg storage unit No. 5 2 strains	100	66.67	0	0	100	33.3	33.3	33.3	0	0	0	100	100	0

14 different antibiotics were tested for expanding the antibiotic resistance profiles among the isolated *E. coli*. The rates of resistance toward the antibiotic panel variated from 20% to absolute resistance (100%). Overall, 83/83 (100%) *E. coli* isolates were resistant to at least two antibiotics, Metronidazole and Tylosine. A total of 49/83 (59.03%) of the *E. coli* isolates were Multi Drug Resistant as they were simultaneously resistant to three or more antibiotics from different classes.

The most MDR pattern was seen with resistance to T 30, TE 30, TY, DO30, STX 25 and MET, while the least frequent pattern was seen with resistance to ENR5, CIP5, NX10 and COT25. The antibiotic resistance rates encountered in three different collection points highlight the spread of resistant bacteria through the farm level. The rates encountered at the egg collection and storage units in each of the studied farms, were similar as those detected in the barns, which reflects the lack of application for the internal biosecurity measures. On the other hand the resistance rates of the *E. coli* strains originating from the feed and egg storage units represented lower resistance rate than those encountered inside the layers barn units. The resistance rate for Ciprofloxacin 5 mcg, not only in the layers barn units but also in the feed and egg storage units, were also concerning regarding the use of this drug in human medicine for *E. coli* infections control.

4. Conclusions

Uncontrolled, avian *E. coli* represents a serious animal welfare concern and risk to public health as it is a zoonotic organism with avian *E. coli* species known to adapt to humans. Salmonellosis is of public health concern because most of the strains of *Salmonella* are potentially pathogenic to humans and animals. Avian salmonellosis can pose a health risk to people if exposed. The antibiotic resistance rates encountered in three different collection points highlight the spread of resistant bacteria through the farm level. The rates encountered at the egg collection and storage units in each of the studied farms, were similar as those detected in the barns, which reflects the lack of application for the internal biosecurity measures. Overall, 83/83 (100%) *E. coli* isolates were resistant to at least two antibiotics, Metronidazole and Tylosine. A total of 49/83 (59.03%) of the *E. coli* isolates were Multi Drug Resistant as they were simultaneously resistant to three or more antibiotics from different classes. The most MDR pattern was seen with resistance to T 30, TE 30, TY, DO30, STX 25 and MET, while the least frequent pattern was seen with resistance to ENR5, CIP5, NX10 and COT25.

Conflicts of Interest: The authors declare no conflict of interest.

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