STUDY OF ANTIBIOTIC RESISTANCE ON *ESCHERICHIA COLI* IN COMMERCIAL POULTRY OF NEPAL

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ABSTRACT

The continuous use of antibiotics in compound feed at sub-therapeutic level has been an integral part of commercial poultry production in Nepal, which is one of the factors that promotes bacterial resistance. Hence, with the objective to determine antibiotic resistance in commercial poultry of Nepal, this study was designed taking Escherichia coli as a flagship bacterium. The commercial layers and broilers birds brought to veterinary teaching hospital of Agriculture and Forestry University by commercial poultry producers for disease diagnosis and treatment were considered as clinical examination of birds were carried out followed by post mortem examination (PME). Those layer/broiler birds which were not taking antibiotic orally or parenterally for last 2 weeks and diagnosed with collibacillosis on PME were included in sampling frame. Air sacculitis, fibrinous pericarditis, fibrinous perihepatitis, and coligranuloma were major criteria for presumptive diagnosis of colibacillosis on PME. The first 40 for both broiler and layer birds totaling 80 that fulfilled the criteria were selected as samples, each representing a commercial farm. All necessary information on daily management practices and previous treatments were obtained from farmer's record book or sheets or face to face interview. Avian pathogenic E. coli was isolated from aseptically collected liver samples and confirmedby biochemical tests. Antibiogram of the isolates were investigated by means of Kirby-Bauer disc diffusion method. E. coli was isolated from all liver samples taken for the culture. It was found that E. coli were resistant most substantially towards Cephalexin (81.2%) and Amoxycillin (81.2%) followed by Tetracycline (78.8%), Colistin sulphate (n=50, 62.5%). Chloramphenicol (61.2%), Ciprofloxacin (55.0%), Enrofloxacin (53.8%), Levofloxacin (28.8%), however, no resistance was found against amikacin. The proportion of E. coli isolates that were resistance against Colistin sulphate ($p<0.05$), Chloramphenicol ($p< 0.05$), Tetracycline ($p<0.001$), Ciprofloxacin ($p<0.01$), Enrofloxacin ($p<0.05$) and Gentamicin ($p<0.01$) were significantly higher in layers compared to that of broilers. In conclusion, avian pathogenic E. coli were resistant towards several antibiotic molecules commonly used in commercial poultry of Nepal, and the resistance was higher in layers compared to broilers.

Keywords: antibiotic resistance, *E. coli*, poultry

INTRODUCTION

The use of antibiotics in compound feeds has been an integral part of poultry production, not only to prevent infectious bacterial diseases but also to promote growth of host. Almost all poultry feed industry in Nepal commonly use different molecules of antibiotics as feed additives in compound feed, pelleted or mash, with the objective to enhance growth and feed efficiency. This continuous use of several types of antibiotic molecules at sub therapeutic level in feeds promotes the bacterial resistance in poultry (Aryal, 2001). The bacteria acquire resistance to particular class of antibiotic either due to de novo mutation, or due to transmission of acquired resistance from one bacterium to others, within species or between species via extra chromosomal DNA (Tenover, 2006). Resistant bacteria thwart antibiotics by interfering their mode of action such as synthesis of inactivating enzymes, alteration in configuration of cell wall or ribosome, and modification of membrane carrier systems, (Timoney *et al*., 1988; Prescott and Dowling, 2013), inhibition of nucleic acid synthesis, inhibition of metabolic pathway (Tenover, 2006). The widespread use of antibiotics as feed additives for growth promotion or disease prevention in food animals could have negative implications for human health and the environment (Hasan *et al.,* 2011).

They also noted a common way of transmission of resistant clones and resistance plasmids of *E. coli* from poultry to human. The development of antibiotic resistance in human bacteria and its association with sub-therapeutic use of antibiotic in animal feed were discussed since early 70's (Dibner and Richards, 2005). The *E. coli* resistant isolates from poultry in a region plays important role for human *E. coli* infections in that region as Agabou *et al.* (2016) showed a clonal and epidemiological link between chicken and human ciprofloxacin-resistant *E coli* isolates in Algeria. Furthermore, the intestine of poultry consists zoonotic potential *E. coli,* which can be transferred from birds to human (Ewers *et al*., 2009). The continuous use of antibiotics in compound feed creates a selective pressure to develop resistant *E. coli* in poultry gut that could make its way in human food chain (Diarra *et al*., 2007). Additionally, in birds, the resistance transferred from generation to generation as pyramid. In a recent study at Sweden, scientists reported occurrence of *E. coli* resistant to nalidixic acid in grandparent birds imported to Sweden for breeding purpose and resistance transferred to parents and their broilers. Similarly, quinolones resistant *E coli* can be introduced from imported breeding birds and spread by vertical transmission (Borjesson *et al*., 2016).

In Nepal, monitoring of antibiotic resistance in veterinary medical field started very late (Khatiwada, 2011) and only a limited number of papers have been published. Antibiogram study of *E. coli* isolated in Nepal from apparently healthy and diarrheic ducks were studied by Singh *et al*., (2013). Shrestha *et al*., (2010) first reported a finding on antimicrobial resistance of Salmonella in poultry of Nepal. In human medical field in Nepal, a couple of studies have been conducted to understand antibiogram of *E. coli* isolated form patients of urinary tract infection (UTI). Sharma *et al*., (2013) and Baral *et al*., (2012) found *E. coli* as one of the major causes of UTI. Majority of *E. coli* showed a resistance towards ampicillin, cephalexin, Nalidixic acid and ciprofloxacin and are multidrug resistant.

In most of the researches, antimicrobial resistance profile of *E. coli* is studied in apparently healthy poultry where *E. coli* are isolated from faecal samples. However, in this research, the antimicrobial resistance was studied in birds which suffered from colibacillsis and *E. coli* was isolated from liver but not from faeces. In this sense, this research is unique and novel. Here, the focus is on the antimicrobial resistance pattern of *E. coli* isolated from birds infected with avian pathogenic *E. coli.* A continuous nation wise surveillance of antimicrobial resistance of *E. coli* could be a major step to understand the pattern of resistance and combat treatment failure in poultry. So, with the objective to evaluate antibiotic resistance profile in commercial broilers and layers, this study was designed taking avian pathogenic *E coli* as a flagship bacterium.

MATERIALS AND METHODS

This research was conducted in Veterinary Teaching Hospital (VTH) and postgraduate laboratory of Department of Veterinary Medicine, Agriculture and Forestry University (AFU) of Nepal during 2014 to January 2016.

Study population

All those birds dead or live which were brought to veterinary teaching hospital for clinical and post mortem examination were defined as study population.

Clinical and PM examination, Inclusion criteria and Sample collection

First, a thorough clinical examination of the birds was conducted with special focus on clinical symptoms of colibacillosis such as swollen head, swollen eyelid, diarrheic vent, swollen joints etc. If the birds were live, they were sacrificed by detaching atlato-axial joint through swirling of neck. A team of registered and experienced veterinarians conducted post mortem examination (PME) of dead birds. Air sacculitis, fibrinous pericarditis, fibrinous perihepatitis, and coligranuloma were major criteria for presumptive diagnosis of colibacillosis on PME. Those layer/broiler birds which were not taking antibiotic orally or parenterally forlast 2 weeks before they were brought to hospital and diagnosed with collibacillosis on PME were included in sampling frame. The first 40 birds for both broiler and layer type chicken totaling 80 birds that fulfilled the inclusion criteria were selected to collect liver samples during PME. Here, each bird in the sampling frame represented a commercial farm. All necessary information on daily management practices and previous treatments were obtained from farmer's record book, sheets or face to face interview.

The reason to take equal number of sample for both broilers and layers was to make a comparison. During postmortem examination, the liver samples were collected aseptically and dispatched to Post graduate laboratory of Department of Veterinary Medicine and Microbiology Unit of Veterinary Teaching Hospital, AFU for culture, isolation and necessary biochemical tests.

Bacteriological Examination

The surface of liver sample in petriplate was first sterilized with red hot spatula. The red-hot inoculating loop was first cooled on the sterilized surface of liver and then inserted gently into liver to take a loopful of content as inoculum. The inoculum was cultured in MacConkey agar and Eosine Methylene Blue (EMB) (HiMedia, India). The quadrant streak method, using whole plate, was employed for culture. After inoculation, the media was incubated for at least 24 hours at 37^{o}C and colony was observed. Pink colony on MacConkey and metallic blue sheen in EMB agar were suggestive of *E. coli.* Colonies showing typical *E. coli* characteristics and morphology were transferred to nutrient agar and incubated at 37 ^{o}C for 24 hours. A single colony was used for biochemical test and confirmation. Confirmation of *E. coli* was drawn by positive Indole test, positive Methyl Red test, negative Voges Praskaur Test, and negative Citrate utilization test [IMViC test (+ + - -)] (Quinn *et al.,* 1984, and Prescott & Dowling, 2013). In indole test, pink/red color on the top of broth indicates a positive result for *E. coli*. Methyl red (MR) and Voges Proskauer (VP) test used MRVP broth. Appearance of red color in MR test and pinkish red color in VP test indicates positive for *E. coli*. Appearance of blue color in citrate agar means positive test for *E. coli.*

Antimicrobial susceptibility testing

The isolates of avian pathogenic *E. Coli* from nutrient agar were subjected for antimicrobial susceptibility test (AST) for study of resistance pattern according to protocol of National Committee for Clinical Laboratory Standards (NCCLS, 2003). The *in vitro* antimicrobial susceptibility pattern of *E. coli* was determined in Muller Hinton agar plates by Kirby-Bauer disc diffusion method. For this, at least 20 pure colonies of *E. coli* were taken directly from nutrient agar plate to make a suspension of 1 ml normal saline. The colony was mixed well in the vortex mixture and a slight turbid color was obtained. The suspension was streaked using a sterile, non-toxic swab on an applicator stick, in three directions over the entire surface of Muller-Hinton media (Hi-media, India) to obtain uniform inoculation. The antimicrobial disc (HiMedia, India) was placed onto the agar surface gently using sterile forceps and kept no closer than 24 mm (center to center) which is equivalent to 6 discs per standard 90 mm petri-dish. The medium was incubated aerobically at 37° C for 24 hours. The degree of resistance or susceptibility was studied based on zone of inhibition, and interpretation was inferred as mentioned in (Table 1).

The selection of antimicrobial molecules was based on their frequency and volume of use as additive in compound feed and while treating colibacilosis in poultry. This information was obtained from registered veterinarians working in poultry industries in Chitwan district, veterinary teaching hospital of AFU, avian laboratories, and veterinary drug centers in Chitwan district through either face to face interview or database analysis of veterinary teaching hospital of AFU. The antimicrobial molecules selected were given in (Table 1).

Table 1: Antimicrobials and their concentration tested in disc diffusion method, and their interpretation criteria

Antimicrobial disc	Abbreviation	Concentration /disc	Interpretation criteria of zone of inhibition (mm)		
			Resistance	**Intermediate**	**Sensitive**
Amoxycillin	AC	20 µg	≤13	14-17	≥18
Colistin Sulphate	CL	10 µg	≤10		≥11
Cephalexin	CFX	5 µg	≤15	16-18	≥19
Chloramphenicol	C	30 µg	≤12	13-17	≥18
Tetracycline	T	30 µg	≤14	15-18	≥19
Ciprofloxacin	CF	5 µg	≤15	16-20	≥21
Enrofloxacin	EN	10 µg	≤14	15-17	≥18
Livofloxacin	LE	5 µg	≤13	14-16	≥17
Gentamicin	G	10 µg	≤12	13-14	≥15
Amikacin	AK	30 µg	≤14	15-16	≥17

Statistical analysis

The required data were collected based on clinical signs, post mortem examination of live or dead birds, culture, isolation and antimicrobial susceptibility test. All descriptive statistics were presented as frequency and percentage. The resistance profile was described in frequency and proportion. The association of antimicrobial resistance of *E coli* with other factors was studied through Pearson's chi square test and Fisher's exact test (for the frequency less than 5). The data analysis was done using SPSS 18.0 version.

RESULTS

The result showed that most of the commercial birds brought to hospitals were Hy-line, Lohman, and H& N for laying hen types and Vancobb- 500 for broiler types. Swollen head syndrome, lameness, synovitis were the characteristic clinical signs and post mortem lesions for diagnosis of colibacillosis. The recognition of presence of these clinical signs and post mortem lesions in in broilers or layers was based on the knowledge and experience of Veterinarians in the research team and standard photographs of lesions. The distribution of these clinical signs and post mortem lesions are given in (Table 2). Significantly, higher proportion of broiler birds were found to suffer from swollen head syndrome, lameness, synovitis compared to that of layer birds. Omphalitis was significantly higher in broiler types, however, coligranuloma was significantly higher in laying hen type birds (Table 2).

The association of type of bird (broilers or layers) with presence or absence of characteristics clinical signs and post mortem lesion (one by one) was studied through Pearson's chi-square test (a 2×2 contingency table for each sign and lesion). The degree of freedom was 1 [(2-1) × (2-1) =1].

Table 2: Distribution of clinical signs and postmortem lesions in birds (Broilers and Layers)

Clinical signs	Bird type		Pearson's χ^2- test
	Broiler (n=40)	Layers (n=40)	p value
Swollen head	21 (52.5)	5 (12.5)	0.00
Lameness	17 (42.5)	3 (7.5)	0.01*
Synovitis	11 (27.5)	1 (2.5)	0.03*
Post-mortem lesions			
Omphalitis	16 (40.0)	0 (0)	NA
Peritonitis	17 (42.5)	23 (57.5)	0.26
Fibrinous pericarditis	34 (85.0)	38 (95.0)	0.63
Fibrinous perihepatitis	31 (77.5)	31 (77.5)	
Coligranuloma	0 (0)	9 (22.5)	0.02*

The value in parentheses are in percent (within the bird type). * Fischer's exact test

NA= The omphalitis was not compared between broilers and layers because it is the characteristics post mortem lesion on broilers only but not in layers. So, there were no any omphalitis in laying hens.

Antimicrobial resistance pattern in poultry

The antibiogram study showed that avian pathogenic *E. coli* isolated from layers had developed resistance to several antibiotic molecules in higher proportion compared to that of broilers. In addition, the avian pathogenic *E. coli* showed relatively higher resistance to cephalexin and amoxicillin compared to other antibiotics both in broilers and laying hen birds. However, we found that *E. coli* had not developed resistance to amikacin both in broilers and layers. The interesting finding, we got in this study was that a proportion of *E. coli* isolated from layers had shown resistance to Gentamicin but none of the *E. coli* isolated from broilers had shown resistance towards Gentamicin (Table 3).

Table 3: Comparison of resistance of *E coli* to different antibiotic molecules in birds

Antimicrobial agent	Antimicrobial resistance in bird type		Pearson's χ^2 test
	Broiler (n=40)	Layer (n=40)	*p-value*
Amoxicillin	30 (75.0)	35 (87.5)	0.275
Colistin sulphate	20 (50.0)	30 (75.0)	0.021
Cephalexin	**31 (77.5)**	**36 (90.0)**	**0.130**
Chloramphenicol	19 (47.5)	30 (75.0)	0.012
Tetracycline	25 (62.5)	38 (95.0)	0.000
Ciprofloxacin	16 (40.0)	28 (70.0)	0.007
Enrofloxacin	15 (37.4)	28 (70.0)	0.004
Levofloxacin	7 (17.5)	16 (40.0)	0.059
Gentamicin	0 (0)	7 (17.5)	0.006*
Amikacin	0 (0)	0 (0)	

The value in parenthesis indicates percent; * Fischer's exact test

The association of antimicrobial resistance of *E. coli* with type of bird (broilers or layers) was studied (one by one for each antimicrobial) through Pearson's chi-square test. A 2×2 contingency table was formed (broilers and layer vs resistance or not resistance). Thus, the degree of freedom was 1 [(2-1) × (2-1) =1].

In our study, *E coli* resistance to Cephalexin was highest (81%, N=80) followed by Amoxycillin (81%), Tetracycline (79%), Colistin sulphate (62%), Chloramphenicol (61%), Ciprofloxacin (55%), Enrofloxacin (54%), Levofloxacin (29%), but there was no resistance against amikacin. The *E. coli* isolate which is resistant to two or more than two antimicrobials were considered multidrug resistant isolates. Substantial proportion of *E. coli* were multidrug resistance. The frequency of multidrug resistant *E. coli* isolated from broilers and layers were highest for Amoxicillin-Cephalexin combination, and Tetracycline and Amoxicillin combination respectively. The details of multidrug antimicrobial resistance profile of *E. coli* isolates are presented in (Table 4).

Table 4: Number of E coli isolates resistance to various antimicrobials

No. of antimicrobials resistant to which *E. coli*	**Combination**	**No. of resistant isolates of *E. coli***	
		Broiler	**Layers**
2	AC-CFX (Broiler) AC-T (Layer)	30	34
3		22	31
4	AC-CFX-T-C	17	26
5	AC-CFX-T-C-CL	14	23
6	AC-CFX-T-C-CL-CF	9	17
7	AC-CFX-T-C-CL-CF-LE (Broiler) AC-CFX-T-C-CL-CF-EN (Layer)	2	8
8	AC-CFX-T-C-CL-CF-LE-G	0	1
9	AC-CFX-T-C-CL-CF-EN-LE-G	0	0
10	AC-CFX-T-C-CL-CF-EN-LE-G-AK	0	0

The resistance profile of *E. coli* for combination with highest frequency are mentioned only. For eg: *E. coli* had highest no of resistance for combination of two antimicrobials (Amoxycillin and Cephalexin) in broiler and that of tetracycline and Cephalexin in layers.

DISCUSSION

The avian pathogenic *E. coli* showed the highest level of resistance to cephalexin in both broiler and layer type birds. The antimicrobial resistance of *E. coli* isolated from layers showed a significant higher proportion for colistin sulphate ($p<0.05$), chloramphenicol ($p< 0.05$), tertracycline ($p<0.001$), ciprofloxacin ($p<0.01$), enrofloxacin ($p<0.05$) and gentamicin ($p<0.01$) in comparison to that of broilers. All the *E. coli* isolated from broilers were sensitive for gentamicin whereas 17.5% (n=7) of isolates in layers had developed resistance to it. *E. coli* isolates from layers mostly showed resistant for most of the antibiotic molecules. The high resistance to several antibiotics in layers might be due to high level of selective pressure because of continuous use of antibiotics since long time in feed. The continuous use of antimicrobial agent creates a selective pressure on bacteria to emerge as a resistant strain (Tenvor, 2006). Dibner & Richards (2005) discussed an epidemiological linkage on prophylactic use of antimicrobials in animal feed and emergence of resistance. World Health Oganization (2000) suggested in the report that animal health management should be routinely practiced avoiding prophylactic use of antimicrobials to as to take precaution to prevent antibiotic resistance. All these reports and finding underpins that the continuous use of antimicrobials is one of the causes to develop resistance in bacteria. This finding coincides with the findings of Karczmarczyk, *et al.,* (2011) where resistance to Tetracycline was the highest followed by Cephalothin (cephalosporin), the Amoxicillin. This finding is also partly supported by the findings of Saidi *et al.,* (2012) where *E. coli* isolates showed moderate rates of resistance to tetracycline and chloramphenicol.

This study showed that most of *E. coli* isolates were multiple drug resistant (MDR) which is in the line of Guerra *et al.,* (2003), Miranda *et al.,* (2008) and Jiang *et al.,* (2011). Antibiotic resistance of avian bacterial pathogens is also a common problem in poultry in Bangladesh. Hasan *et al.,*(2011) noted that more than 55% (N=101) of *E coli* isolated were resistant to at least one or more of the tested antibiotics, and 36% of the isolates showed multiple-drug-resistant phenotypes. The most common resistances observed were against Tetracycline. This supports our finding in the way that the resistance of *E. coli* was highest against tetracycline in laying birds. It also reported moderate resistance to gentamicin which is in same line to our finding.

According to Jiang *et al*., (2011), *E. coli* strains isolated from both apparently healthy (from feces) and diseased (from liver) poultry (chicken, ducks and partridges) (n=389) from China had highest resistance to tetracycline (91%), amoxicillin (40%), and Chloramphenicol (34%). The high rate of resistance towards tetracycline, amoxicillin and chloramphenicol support our finding. They also found substantial proportion of E coli isolates from poultry which were multidrug resistant. Similarly, Zhang *et al.,* (2014) from Hebei of China reported high rate of resistance toward (N=111) gentamicin (95%), amikacin (46%), which is contrast to this study. This can be because of continuous over exploitation of aminoglycosides in growing and finishing poultry in China, however the drugs antimicrobials in the group of aminoglycosides are not commonly used in poultry feed. Samanta *et al.,* (2013) from India reported prevalence of *E coli* in healthy layers and their environment. The report showed that the resistance of the isolates was most frequently observed to chloramphenicol (87%, n= 313). In their study, none of the isolates was found to possess quinolone resistance which is contrast to our finding where substantial proportion of *E. coli* have developed resistant to quinolones (ciprofloxacin, levofloxacin). Several reports of multidrug resistant *E. coli* isolated from apparently healthy or sick chickens or from faecal and liver samples of poultry have been published emanating from African countries (Maine *et al*., 1998; Geornaras *et al*., 2001; Oladele *et al*., 2008,).

One of the most striking factors for antimicrobial resistance in poultry is the haphazard use of antibiotics. The other factors which promotes this resistance might be poor sanitation of barn and hence the crowding of several bacteria in gastrointestinal tract of poultry. The higher level of antibiotic resistance in laying hens could be because of continuous use of antibiotics for longer time compared to that of broilers. All the laying hens in the samples were more than 20 weeks of age and they have been provided with antibiotics in feed since day one old. The higher level of resistance to Amoxicillin and Tetracycline shown in the study could be because of higher use of these antimicrobial molecules in poultry either in feed or via drinking water. Amoxicillin and tetracycline are the most commonly used antibiotic molecules for treatment of poultry in Nepal. Most of the researchers Miranda *et al*., (2008); Rosengren(2008) and Varga *et al*., (2009) and Jiang *et al*., (2011) agreed that there is a direct relationship between antimicrobial resistance and antimicrobial use. The layers birds have longer period of growth relative broilers which results in consumption of higher quantities of a wider range of antimicrobials by laying hens. This could be a reason for higher proportion of antimicrobial resistance of *E. coli* to various antimicrobials (Jiang*et al*., 2011).

The higher proportion of resistant bacteria towards amoxicillin and tetracycline possess a chance of spilling over to other population. These bacteria can transmit the resistance gene to other bacteria to develop the resistance for antimicrobials (Alekshun and Levy, 2007). Till date, the resistance towards amikacin has not been reported in poultry, however, if the use of antibiotic molecules increases in the increasing order, some days in future, the bacteria in poultry can develop resistance substantially even to gentamicin and amikacin. The intestinal flora of poultry can provide a reservoir of antibiotic resistant bacteria that can infect or colonize humans via the food chain.

CONCLUSION

The *E. coli* isolated from broilers were highly resistant to cephalexin followed by amoxicillin, tetracycline, and chloramphenicol. The *E coli* isolated from layers were highly resistant to Tetracycline followed by Cephalexin, Amoxicillin, Ciprofloxacin, Enrofloxacin etc. The *E. coli* isolated from both broilers and layers were sensitive to amikacin. *E. coli* isolated from broilers were not resistant to Gentamicin, however, some proportion of *E. coli* isolated from layers were resistant to Gentamicin. The level of antimicrobial resistance was found higher in *E. coli* isolated from layers relative to broilers. A substantial proportion *E. coli* isolated from both broilers and layers suffered from colibacillosis showed a multi-drug antimicrobial resistance pattern.

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