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Research Article

Antibiogram of *Escherichia coli* Isolated from Avian Colibacillosis in Chitwan District of Nepal

D.B. Thapa[1], A. Chapagain[2*]

[1]Faculty of Animal Science, Veterinary Science and Fisheries, Agriculture and Forestry University, Nepal
[2]Institute of Agriculture and Animal Science, Tribhuvan University, Nepal

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***Corresponding author**
A. Chapagain,
Institute of Agriculture and Animal Science, Tribhuvan University, Nepal.
Email: arjun@iaas.edu.np/ chapagain.arjun@gmail.com

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Abstract

A cross-sectional study was conducted at National Avian Disease Investigation Laboratory, Chitwan to determine antibiogram of *Escherichia coli* isolated from avian colibacillosis cases of broilers and layers in Chitwan. One hundred and sixty (95 from broilers and 65 from layers) liver samples were collected aseptically during postmortem. Samples were taken purposively from dead birds showing lesions perihepatitis, pericarditis, air-saculitis, omphalitis and egg peritonitis. Isolation and identification were made by examination of cultural characteristics of *E. coli* in MacConkey's agar, Eosin methylene blue (EMB) agar, Gram's staining and biochemical tests. Antibiogram of identified *E. coli* isolate was evaluated against six antibiotics of six different groups by disk diffusion method following CLSI guidelines. One hundred and three *E. coli*isolates (73 from broilers and 30 from layers) were isolated from one hundred and sixty samples. Highest resistance was observed against Ampicillin (100%) followed by Co-trimoxazole (86.40%), Doxycycline (46.60%), Levofloxacin (45.63%), Nitrofurantoin (26.21%) and Amikacin (10.68%). Nearly about all (96.12%) isolates from 103 isolated *E. coli* isolates showed multidrugs resistance to two or more than two antimicrobials. All multidrug resistance isolates showed 16 different patterns with each isolate being resistance to at least two drugs. The multiple antibiotic resistance indexing ranged from 0.2 to 0.8 and proportion of isolates with MAR index greater than 0.2 was 96.12%.

Keywords: antibiogram; *Escherichia coli*; colibacillosis; poultry; multiple antibiotic resistance (MAR)

Introduction

Nepal is predominantly an agricultural country where 65.66% people depend on agriculture for their livelihood. Nepalese livestock sector contributes 11% in gross domestic production (GDP) and 26.8% in agricultural gross domestic production; (AGDP) (MOLD, 2016). Contribution of poultry in GDP is 3.5 percent. Total investment in poultry is NRs 22 billion (FAO, 2014). In national level total meat production per year is 303000 metric tons (MOLD, 2016), among which chicken meat contributes 14.46% of total meat production. Poultry industry has been developed as one of the commercial enterprises in the private sector of Nepal. Chitwan district is major poultry pocket of Nepal. In national total production of broilers and eggs, Chitwan contributes 10% in broilers and 68% in egg production (CBS, 2016).

E. coli is gram negative, non-spore forming, rod shaped (1.1 – 1.5 µm by 2.0-6.0) micro-organism often motile by means of flagella or may be non-motile, and which can grow with or without oxygen (Fratamico and Smith, 2006).

Colibacillosis refers to any localized or systemic infection caused entirely or partly by Avian Pathogenic *Escherichia coli* (APEC), including colisepticemia, coligranuloma (Hjarre's disease), air sac disease, chronic respiratory disease, (CRD), cellulites (inflammatory process), swollen-head syndrome, peritonitis, salphangitis, osteomyelitis/synovitis (turkey oeteomyelitis complex), panophthalmitis, and omphalitis/yolk sac infection (Saif, 2003). Colisepticemia is the most common form of colibacillosis and is responsible for significant economic losses in aviculture in many parts of the world (Ewers *et al.*, 2004).

Recently there has been rampant use of antimicrobials as growth enhancer as well as treatment for diseases, has resulted in multidrug resistance microbes such as *E. coli*, *Salmonella* and *Staphylococcus aureus.* These resistant microbes are causing failure of treatment and transmission of resistant pathogens to humans with zoonotic importance which is also the next emerging problem (CDC, 2013).

In our context, very limited data regarding antimicrobial use pattern in food animals are available. It is realized that data on use of antimicrobial in food animals are very essential for identifying and quantifying the risk of developing and spreading antimicrobial resistance in food chain. Researches in the field of detection of antimicrobial resistant against certain common disease-causing organisms are lacking.

The study was aimed to find out the antibiogram of the isolates of *E. coli* strains from visceral organs (liver and heart) of poultry of Chitwan suspected for colibacilosis brought in National Avian Disease Investigation Laboratory (NADIL) for assessing their susceptibility and resistance patterns to some selected antimicrobials.

Material and Methods

Liver samples from diseased and dead broilers and layers were collected from Chitwan and brought to National Avian Disease Investigation Laboratory (NADIL), Bharatpur, Chitwan.Tissue (liver) were collected based on clinical finding and pathogonomic lesions observed during detailed post mortem examination of poultry at postmortem unit of NADIL. Livers were collected from cases exhibiting perihepatitis, pericarditis, air sacculitis and yolk sac infection. Samples were collected into sterile Petri disc in postmortem unit and immediately transported in microbiology unit.160 (broilers 95 and layers 65) liver samples were collected based on the formula given by (Thrusfield, 2007). Cross sectional study was done for the purpose.

All the required media were prepared just prior to processing of the samples. The instructions given by HiMedia were followed carefully.

Isolation and Identification of E. coli

Surface of organ was seared by hot spatula, and then sterile microbiological loop was introduced deeply in the affected organs, then loopfuls was inoculated into selective media (MacConkey's agar) and incubated aerobically at 37^{o}C for 24 hours. Bacterial colonies that were rose pink in colour, 2-3 mm in diameter, opaque and convex with entire edge and lactose fermentative on MacConkey agar were further streaked into Eosine Methylene Blue (EMB) and incubated overnight at 37^{o}C. Colonies on EMB agar with green metallic sheen were suspected as positive for *E. coli* and were confirmed by biochemical tests and for pure culture organism was sub cultured in TSA.

Biochemical tests include gram stain, IMViC test *i.e.* indole, methyl red, Voges-Proskauer and citrate utilization tests, oxidase test, catalase test and growth on TSI agar for the confirmation. Suspected bacterial colonies were confirmed as *E. coli* by negative gram stained rod, positive MR and indole test and catalase test, negative VP, citrate and oxidase test and yellow slant and butt, positive gas and negative H_2S production in TSI.

Morphological Characterization by Gram's Staining Method

A small colony was picked up with a bacteriological loop, smeared on a glass slide and fixed by gently heating. Crystal violet solution was then applied on the smear to stain for two minutes and then washed with running water. Lugol's iodine was then added to act as mordant for one minute and then again washed with running water. Acetone alcohol was then added, which act as a decolorizer, for 5 seconds. After washing with water, Safranine was added as counter stain and allowed to stain for two minutes. The slide was then washed with water, blotted and dried in air and then examined under microscope with high power objectives (100X) using immersion oil (Merchant and Packer, 1967).

Biochemical Test

Methyl Red – Voges-Proskauer test (MR-VP test)

The sterilized tube containing 4ml of MR-VP medium was inoculated with loop full of colonies and incubated at 37^{o}C for 48 hrs. After incubation, equal amount of MR-VP broth was transferred to two small sterile tubes. One tube for methyl red (MR) test and the other for the Voges-Proskauer (VP) test. MR-VP test carried out as described by Barrow and Felthan (1993).

MR Test

Five (5) drops of methyl red was added to one tube, shaken well and examined. Presence of red colour indicates positive result whereas yellow colour indicates negative result.

VP Test

It was used to test the production of acetyl-methyl-carbinol from glucose. The test culture was inoculated into next tube containing MR VP medium and incubated at 37ºC for 48 hours. Then 0.6 ml of 5% α- naphthol followed by a 0.2 ml of 40% potassium hydroxide aqueous solution per one ml of culture were added to the culture, shaked well and examined after 5 to 15 minutes. A positive reaction was indicated by appearance of bright pink colour.

Oxidase Test

Method of Barrow and Felthan (1993) was used. Strip of filter paper was soaked in 1% solution of tetra methyl-p-phenylenediamine dihydrochloride and dried in hot air oven and then placed on clean glass, slide by sterile forceps. A fresh young tested culture on TSA was picked off with sterile glass rod and rubbed on filter paper strip. If a purple color developed within 5-10 seconds, the reaction was considered positive result whereas absence of purple colour gave negative result.

Catalase Test

A drop of 3% aqueous solution of hydrogen peroxide was placed on a clean slide. Small amount of tested organism colony on nutrient agar was picked by glass rod, added to the drop and mixed. Positive result was indicated by presence of immediate bubbling or foaming and liberation of O_2 gas and its absence was indicated negative result (Barrow and Felthan, 1993).

Citrate Utilization Test

Pure colonies were inoculated by making a streak onto the surface of slant of simmon citrate agar prepared according to manufacturer's instruction and incubated at 37ºC for 24 hrs and slants were inspected. Presence of blue-green colour in the medium was positive result and no colour change *i.e*. remaining dark green colour in the medium was negative result (Quinn *et al.*, 2002).

Table 1: Interpretation of TSI test results

Slant/Butt	Colour/Reaction	Interpretation
K/N or K/A	Red/orange(oxidative) or Red/yellow(fermentative)	Only peptone utilized or only glucose-fermented
A/A	Yellow/yellow Splitting or bubbles	Glucose, plus lactose
Gas	Black butt	Gas production
H_2S		Hydrogen sulfide production

Triple Sugar Iron (TSI) Test

TSI slants were prepared according to manufacturer's instructions. Sterile needle with pure colonies was inoculated into TSI slant by stabbing to the bottom of the tube and then streaking the surface of the slant as the needle is drawn out of the tube and incubated at 37ºC for 24 hours (Quinn, *et al.*, 2002). Interpretation of result has been shown in Table 1.

Reaction of The Organism in TSI Agar Slant

Yellow slant, yellow butt, presence of gas bubbles and absence of black precipitate in the butt (due to the production of H_2S) indicative of *E. coli* (Carter, 1986).

Motility – Indole – Ornithine Test (MIO Test)

Test was performed as described by Quinn *et al* (2002). Test was performed in a single tube; following overnight (18–24 hour) incubation, motility and ornithine decarboxylase activity was determined by visual examination. After the motility and ornithine decarboxylase results were interpreted, indole results were interpreted following the addition of Kovac's reagent. A small amount of growth was harvested with a sterile inoculating needle. A single stab was made in the tube of semisolid MIO agar which had been prepared according to manufacturers' instructions. It was made straight into the agar stopping approximately 1cm from the bottom of the tube. Tubes were incubated under aerobic conditions at 37ºC for 18- 24 hours caps loosened. Observations were made and interpreted as follow:

Motility Test

Positive: visible growth extending away from the stab line. Typically, the agar will become visibly turbid.

Negative: growth only along the stab line. The agar remains clear. Isolates which only produce small tufts of growth a long stab line (similar to bristles on a brush) are considered non–motile.

Ornithine Decarboxylase Test

Positive: Agar in the middle of the tube turns a light purple colour. These tubes are distinctly purple; however, they will be a lighter shade of purple than their uninoculated counterparts.

Negative: Agar in the middle of the tube turns yellow. Only the colour of the agar in the middle of the tube should be noted. Oxidation may cause the agar on the surface of the tube to turn purple, this is not significant. The above two, are recorded prior to the next step, because Kovac's reagent will cause the agar to turn yellow.

Indole Test

Three to four drops of Kovac's reagent are added to the surface of MIO medium in tube.

Positive: Kovac's reagent turns pink – red, red ring at interface of medium.

Negative: No colour change is observed that is Kovac's reagent remains orange yellow.

Antimicrobial Susceptibility Testing (AST)

After biochemical conformation, single colony was sub cultured in TSA for antimicrobial susceptibility testing.

AST was done by the standard Kriby-Bauer disk diffusion method following guidelines provided by CLSI (2009).

Data Analysis

Data entry and analysis was done using program Microsoft Office Excel 2007. The result of microbiological examination (positive and negative) and antimicrobial susceptibility test resultsof *E. coli* isolates in broilers and layers were analyzed statistically by using chi square and Fishers exact test with significance level defined at the $p < 0.005$ using commercial software Graphpad Prism 6. Multiple Antibiotic Resistance (MAR) Index was calculated using data of antimicrobial susceptibility testing. MAR Index calculated as a/b (Krumperman, 1983), where 'a' is number of resistance antibiotics and 'b' is number of antibiotics used.

Results

Out of 160 samples taken from broilers (95) and layers (65), 103 (64.37%) isolates were found positive for *E. coli* and the rest as negative.

Moreover, out of 95 broilers samples, 73 (76.84%) number of *E. coli* were isolated and out of 65 layers samples, 30 (46.15%) number of *E. coli* were isolated (Table 2).

Statistical analysis showed that there was a significant difference between broilers and layers (p-value<0.05).

Table 2: Species wise positive and negative isolate on culture basis

Species	Total Samples	Positive	Negative	p-Value
Broilers	95	73 (76.84%)	22 (23.16%)	<0.001
Layers	65	30 (46.15%)	35 (53.85%)	

Antibiogram profile of 103 *E. coli* isolates showed highest resistance against Amikacin (100%) followed by Co-trimoxazole (86.40%), Doxycycline (46.60%), Levofloxacin (45.63%), Nitrofurantoin (26.21%) and Amikacin (10.68%). Doxycyclin (33%) showed highest intermediate resistance. Highest sensitivity was against Amikacin (88.35%) followed by Nitrofurantin (55.34%). All isolates were resistance against at least one antimicrobial agent. Overall antimicrobial sensitivity pattern of *E. coli* isolates are shown in Fig 1.

Data shown in Table 3 shows that resistance pattern is similar between layers and broilers which is highest in Amikacin followed by Co-trimoxazole. Doxycyline is more resistance in broilers than layers samples but Levofoxacin was more resistance in layers than broilers sample. Sensitivity pattern is also similar between both samples.

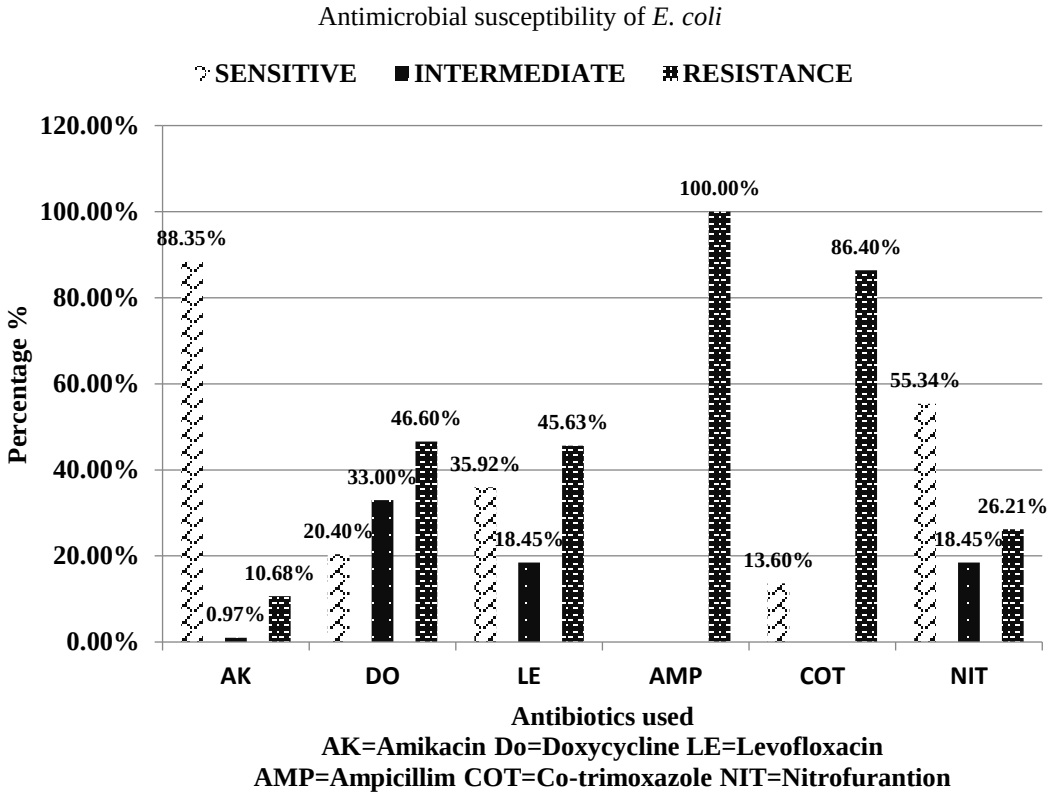

Fig. 1: Overall antimicrobial resistance pattern of *E. coli isolates* from both broilers and layers

Table 3: Antibiotic resistance pattern of broilers and layers.

Name of Antibiotics	Broilers Resistance %	Layers Resistance %	p-value
Amikacin	12.33	6.67	0.5006
Ampicillin	100	100	1.0000
Co-trimoxazole	83.56	93.33	0.2183
Doxycycline	17.80	53.33	0.1827
Levofloxacin	34.25	40	0.7613
Nitrofurantion	53.42	26.67	0.6807

Statistically there was no significant difference between the resistance pattern of *E. coli* isolated from broilers and layers i.e. p-value is > 0.05.

Table 4: t-Test: two samples assuming equal variance for Mean Disc Diffusion Zone Diameter for *E. coli* isolated from Broilers and Layers

Antimicrobials	Broilers Mean±SE (mm)	Layers Mean±SE (mm)	p-value
Amikacin	19.52±0.371	19.47±0.338	0.930
Ampicillin	6.35±0.114	6.367±0.162	0.959
Co-trimoxazole	9.904±0.979	8.167±1.028	0.299
Doxycycline	11.48±0.401	10.60±0.520	0.217
Levofloxacin	15.47±0.683	15.47±0.796	0.999
Nitrofurantion	16.56±0.330	17.07±0.544	0.419

Species wise comparison of mean disc diffusion diameter of *E. coli* isolated from broilers and layers showed no significance difference between them i.e. p-value > 0.05. Highest zone of inhibition shown by Amikacin (19.50±0.28 mm) followed by Nitrofurantoin (16.71±0.28 mm), Levofloxacin (15.47±0.53 mm), Doxycycline (11.22±0.32 mm) and Co-trimoxazole (9.40 ±0.76). Ampicillin (6.36 ±0.09) showed lowest zone of inhibition. Mean zone of inhibition for each antibiotic is given in Table 3.

Mean and standard error of mean is shown in Fig. 2 (AK-Amikacin, DO-Doxycycline, LE-Levofloxacin, AMP-Ampicillin, COT-Co-trimoxazole, NIT-Nitrofurantoin)

Multiple antibiotic resistances are resistant shown by organism against two or more antimicrobial agents (Fig. 3). 99 (96.12%) isolates from 103 isolated *E. coli* isolates showed multidrug resistance to two or more than two antimicrobials. All multidrug resistance isolates showed 16 different patterns with each isolate being resistance to at least two drugs. The highest levels (36.89%) of multidrug-resistant *E. coli* were observed for 3 antimicrobials (Table 5).

Table 5: Multiple antibiotics resistance patterns of *E. coli* isolates

No. of antibiotics	Resistance pattern	No. of isolates
1	AMP	4
2	AMP+COT	18
	DO+AMP	3
3	DO+AMP+COT	10
	LE+AMP+COT	15
	DO+LE+AMP	3
	AMP+COT+NIT	3
	AK+DO+AMP	1
	DO+AMP+NIT	2
	AK+AMP+COT	5
4	DO+LE+AMP+COT	13
	DO+AMP+COT+NIT	9
	AK+LE+AMP+COT	2
	LE+AMP+COT+NIT	8
	AK+DO+LE+AMP	2
	AK+DO+COT+AMP	1
5	DO+LE+AMP+COT+NIT	4

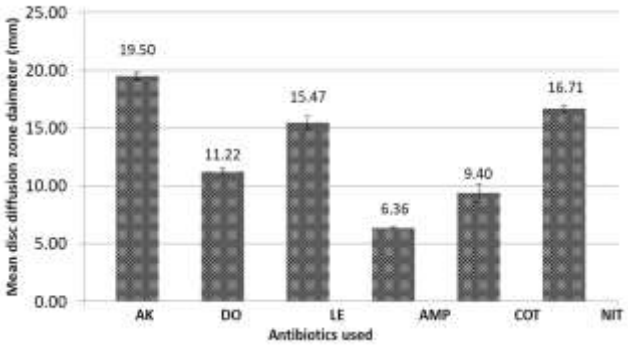

Fig. 2: Mean zone of inhibition of various antibiotics (mm) against all *E. coli* isolates

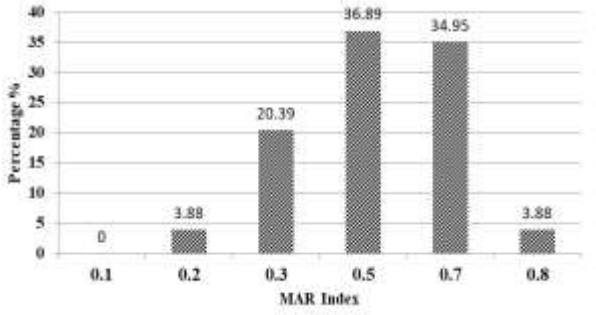

Fig. 3: Multiple antibiotic resistance indices of *E. coli* isolates

Proportion of isolates with MAR index greater than 0.2 was 96.12%. High portion of isolates 36.89% showed MAR index 0.5 and 35.95% of isolates showed MAR index 0.66.

Discussions

Present study attempted to determine the prevalence of *Escherichia coli* among dead poultry which has significant role in causing disease and these are being increasingly detected among poultry flocks. This study showed more than half (64.37%) of the samples contained *E. coli* isolated that seems similar to the isolation rate by Ammar *et al.*, (2015) isolated 63 percent. Likewise, Samanta *et al.*, (2014), isolated *E. coli* with percentage of 75% from backyard chicken in India which are similar to isolation rate in broilers but higher than isolation rate in layers in compare with this study. However, Joshi *et al.*, (2012) reported isolation rate 57% in layers which is greater than isolation rate in layers of current study and Boukhors *et al.* (2013) isolated 54% *E. coli* form liver samples of broilers which is lower than isolation rate in broilers of this study.

Ampicillin (penicillin), Amikacin (aminoglycosides), Doxycycline (tetracycline), Levofloxacin (flouroquinolones), CO-trimoxazole (sulphonamides and trimethoprim) and Nitrofurantoin (Nitrofurans); six antibiotics disc of six different groups of drugs were used for Antimicrobial Susceptibility Testing (AST). One hundred and three *E. coli* liver samples were used for AST that shows highest resistance against Ampicillin (100%) this study shows similarities with research done by Geidam *et al.*, (2012) *i.e.* Ampicillin was not at all effective. Shrestha *et al.*, (2011) and Al-Afraj *et al.*, (2015) reported Ampicillin 100% resistance in *E. coli* from poultry which seems similarities with this study. Whereas Co-trimoxazole (86.40%), Doxycycline (46.60%) and Levofloxacin (45.63%) are resistant.

This study shows Levofloxacin was found 45.6% resistant. Antibiogram of *E. coli* isolated from human urine sample by Zinnah *et al.*, (2008) showed 40% resistance, 20% intermediate and 40% resistant against Levofloxacin which is similar to this finding and match with the resistant pattern of layers's isolates. Higher resistance 91.93% against Levofloxacin was observed by Priti and Satish (2014). Another study conducted in Veterinary teaching hospital, Rampur by Neupane *et al* (2005) reported resistance against Levfloxacin is 28.8% which is about 17% less than present study.

Resistance of Co-trimoxazole (sulphamethoxazole+trimethoprim) was found 86.40% and got similarities of finding of Balasubramaniam *et al.*, (2014) 83%, Salehi and Bonab (2006) 80% and Bukhrosh and Hamdi (2013) 82.2 %. Haghighi and Peighambari (2005) also reported 94% resistance in COT which is also similar to this study.

Resistance of *E. coli* against Doxycycline was 46.6% isolates which agreed with Raum *et al.*, (2008) he stated 29–58% resistance of *E. coli* to Doxycycline isolated from stool samples in a study in Germany. Resistance of Doxycycline was close to study by Hassan *et al.*, (2014) 53.75%. But higher resistance against Doxycycline was observed 98.3% by Bukhrosh and Hamdi (2013) and 80% by EI Tawab *et al.*, (2016).

Resistance exhibited by Nitrofurantoin in this study was 26.03% which is similar to the finding of Atere *et al.*, (2015) and Bukhrosh and Hamdi (2013) showed 20.8% and 18.9% resistance but higher (56%) resistance was found by Salehi and Bonab (2006). Antibiogram investigation of the *E. coli* isolates from pigeons by Dutta *et al.*, (2013) revealed that 100% isolates exhibited resistant against Ampicillin which is similar to this study but relatively higher resistant exhibited in Nitrofurantoin 73.62%. Sensitivity of Nitrofurantoin in this study was observed 55.34%. Which is closer to Pachaury and Kataria (2013) 66.95% and Tiltilawo *et al.*, (2015) 64%.

Radwan *et al.*, (2014) showed antibiogram profiles of *E. coli* indicated maximum resistance to Ampicillin (100%) and Sulfamethoxazole/trimthoprim (94%). Conversely, the aminoglycoside amikacin was still effective against 97.6% of the isolates which is similar to finding of this study which and highest sensitive found in Amikacin 88.35%. Neupane *et al.* (2005) reported no resistance against Amikacin in poultry isolates and 90% sensitive showed by Bist *et al.*

(2014). Result of sensitivity of Amikacin of this study is contrast to study of Priti and Satish (2014) and Balasubramaniam (2014) in which resistance of Amikacin was 88.33% and 71% respectively. Resistance against Amikacin was observed 10.68% in this study. Joshi *et al.*, (2012) also reported lower resistance against Amikacin 5.26%.

Mean zone of inhibition of Amikacin, Doxycycline, Levofloxacin, Ampicillin, Co-trimoxazole and Nitrofurantoin were 19.50 mm, 11.22 mm, 15.47 mm, 6.36 mm, 9.40 mm and 16.71 mm respectively. Study conducted by Shrestha *et al.*, (2011) in Chitwan reported in overall poultry isolates mean zone disk diffusion diameter of Ampicillin, Co-trimoxazole, Doxycycline and Nitrofurantoin were 6.1mm, 12.85mm, 12.71mm and 16.1 mm respectively which is similar finding with this study.

This study showed 96.12% isolates were resistance at least for 2 antibiotics which is similar with finding of Zeryehum and Berhanu (93.2%). Olarinmoye *et al.*, (2013) observed 84.68% were MDR phenotypes where MAR Index $\geq$ 0.2. Hamisi *et al.*,(2014) found 80.59% isolates were MDR isolated from free range chickens and 19 MDR pattern were reported. Study of Hassan *et al.*, (2014) exhibited all of the isolates showed multiple antimicrobial resistances. Studies of Bukhors and Hamdi, (2013) 100%, Cunhna *et al.*, (2014) 92%, Radwan *et al.*, (2014) 90.4%, Rahimi (2013) 63.3% exhibited higher prevalence of MDR *E. coli* in poultry. Other observations also demonstrated a similar finding on multiple drugs resistance of *E. coli* isolates (Salehi and Bonab, 2006; Guerra, 2007; Akond, 2009; Majalija *et al.*, 2010). Proportion of isolates with MAR index greater than 0.2 was 96.12%. MAR index values greater than 0.2 indicate high risk source of contamination, where several antibiotics are often used (Tambekar *et al.*, 2006). These showed administration of multiple antibiotics for prophylaxis or infection. Furthermore, it's a strong indication of abusive and indiscriminant use of antibiotics in the farms. Such multi-drug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antibiotic saturated environment (Van De Bogaard and Stobberingh, 2000).

This study showed a high prevalence of antimicrobial drug resistance in *E. coli* isolated from poultry. Resistance recorded to Ampicillin in present study might be due to β-lactamase production by the isolates. In addition, indiscriminate use of antibiotics exerts a selection pressure which leads to development of drug resistance in the isolates. One reason that could explain high prevalence of resistance in *E. coli* to these antimicrobials could be because of their frequent use in live chicken for therapeutic purposes as well as enhanced growth promotion.

Conclusion

This study showed resistance against the antibiotics that are commonly used in poultry. These findings confirm significant increase in the incidence of antimicrobial resistance in *E. coli* isolates which is most probably due to increased use of antibiotics as feed additives for growth promotion and prevention of diseases and use of inappropriate antibiotics for treatment of diseases. Hence, excess or abusive use of antimicrobials should be guarded through judicious application of antimicrobials.

Author's Contribution

The both authors are equally involved in finalizing the research article from research design, sample collection, research work, and data analysis to preparation of manuscript.

Conflict of Interest

There is no any conflict between authors from sample collection to publications of manuscript.

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