Journal of Zoonotic Diseases
2021, 5(1): 18-28
doi: 10.22034/jzd.2021.12711
https://jzd.tabrizu.ac.ir/article_12711.html

Original Article

Epidemiology and antimicrobial resistance of pathogenic *E. coli* in chickens from selected poultry farms in Zambia

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(Received 15 March 2021, Accepted 7 April 2021)

Summary
Escherichia coli (E. coli) bacteria are one of the most important pathogens in the poultry industry and a leading cause of cellulitis, septicemia, and airsacculitis infections. Antimicrobial resistance in pathogenic *E. coli* is of particular interest because it is the most common gram-negative pathogen in chickens. Cloacal, eggs, and environmental samples were randomly collected from three commercial farms in Zambia. Specimens were cultured and phenotypically identified pathogenic *E. coli* using Congo red dye-binding test (CR-test). The pathogenic *E. coli* underwent antimicrobial susceptibility testing for six antibiotics. The study aimed to isolate and determine antimicrobial resistance patterns of pathogenic *E. coli* from chickens in Chisamba and Lusaka districts. A total of 417 samples were collected and processed microbiologically. *E. coli* was isolated from 333(79.9%; 95%CI=75.23-82.98) samples. The highest number was isolated from cloacal swabs 313(75.1%; 95%CI=70.19-78.52%) while 18(4.3%; 95%CI=2.75-6.72%) was from litter in poultry houses, and 1(0.2%) of each from eggs and environment swabs. Of 333 isolates, 62(18.6%; 95%CI=14.90-23.28%) were pathogenic. The bacteria demonstrated 100% and 92% resistance to tetracycline and cephalexin, respectively, while 77% were susceptible to gentamicin. The results also showed that 4.8% of pathogenic isolates exhibited multidrug resistance (MDR) to all six antibiotics combined, while 17.7% were resistant to five antibiotics. The isolation of antimicrobial-resistant pathogenic *E. coli* suggests that the bacteria were exposed to these antibiotics before sampling. The resistant bacteria are a serious public health concern, causing ailments that are difficult to treat with antimicrobial drugs. Consequently, there is a need to intensify education campaigns on biosecurity measures and good-hygienic practices.
Keywords: Antimicrobial resistance, Chicken, Pathogenic *Escherichia coli*.

Introduction

Escherichia coli (*E. coli*) is a member of the genus *Escherichia* that contains mostly motile gram-negative, rod-shaped bacteria of the family *Enterobacteriaceae* (Filho et al., 2015). *E. coli* is present as the microbiota (commensal bacteria) in the intestinal tract of the poultry. The bacteria could be either non-pathogenic or pathogenic causing an infection known as avian pathogenic *Escherichia coli* (APEC) also known as

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colibacillosis. This is an infectious disease leading to acute fatal septicemia or sub-acute fibrinous pericarditis, airsacculitis, peritonitis, and salpingitis in broiler chickens aged 4–6 weeks. It also causes egg yolk retention in laying birds and omphalitis in chicks (Munang'andu et al., 2012; Ahmad et al., 2009). The disease decreases egg productivity and increases mortality, prophylaxis, and treatment costs resulting in economic loss in the poultry industry (Ali and Al-Mayah, 2016; Zhuang et al., 2014). The presence of pathogenic microorganisms in poultry meat and/or its by-products remains a significant concern to consumers and public health officials worldwide. The bacteria has been consistently associated with food-borne illnesses in most countries (Kabir, 2010).

The disease has not spared Africa and reported in countries such as the Democratic Republic of Congo (DRC), South Africa, Swaziland, Central African Republic, Kenya, Uganda, Gabon, Nigeria, and Ivory Coast (Kanengoni et al., 2017; Raji et al., 2006; Effler et al., 2001). In Zambia, the disease had been reported in domestic cattle, pigs, and poultry (Mainda, 2016). It is associated with food-borne and water-borne transmission, while person-to-person transmission has also been reported (Raji et al., 2006). The inanimate objects such as gumboots, clothes, and vehicles also have been associated with the transmission of the disease within the farm and the communities (Mainda, 2016). These disease conditions are managed by the treatment of sick birds with antibiotics. The widespread non-human use of antibiotics is regarded as highly essential for use in animal production, which promotes a reservoir of resistant bacteria and resistance genes to produce multidrug-resistant (MDR) bacteria (Ishiguro et al., 1978). This practice undoubtedly may add to the burden of antimicrobial resistance (AMR) in human medicine and truncate the period that these valuable antimicrobial agents will be operative for such infections (Hammerum and Heuer, 2009). AMR is a serious challenge not only in Zambia but also throughout the world. Humans may obtain antimicrobial-resistant *E. coli* or resistance genes of animal origin directly via contact with animals, food of animal origin, and the environment (Skurnik et al., 2015). The bacteria may also acquire drug resistance genes through antibiotics use in livestock feed at low doses for growth promotion. The bacteria may also be exposed to antibiotic agents when pharmaceutical companies release quantities of antibiotics into the environment. Human beings indirectly may be exposed to antibiotics when they use soaps and other related products impregnated with antibacterial agents (D'Costa et al., 2011; Laxminarayan et al., 2013; Ferber, 2002). A study by Mshana reported an increasing trend of resistance to commonly used antibiotics namely ampicillin, co-trimoxazole, gentamicin, erythromycin, tetracycline, and third-generation cephalosporin (Mshana et al., 2013). The antibiotic resistance to some drugs could develop as a result of the resistance genes such as plasmid-mediated *Tet* genes in Tetracycline and extended-spectrum of beta-lactamases (ESBL) in most penicillins (Zibandeh et al., 2016). Therefore, it's essential to monitor the use of antibiotics and antimicrobial resistance in these bacteria.

Diagnosis of *E. coli* infection is based on observing clinical signs and laboratory investigation of the sick birds and appropriate samples, respectively. Laboratory diagnosis of *E. coli* is usually by culture using Mac Conkey agar, Eosin Methylene Blue (EMB) agar, biochemical, and molecular tests, which may probably be expensive. The ability to distinguish between pathogenic (invasive) and non-pathogenic *E. coli* is an essential parameter for monitoring virulence physiognomies of the bacteria in the human and animal communities which can be achieved by the use of the Congo red dye agar test (CR test). The invasive or pathogenic strains bind to the dye and produce red colonies within 72 hours of incubation (Sharma et al., 2006). This study aims to investigate the prevalence and antimicrobial resistance (AMR) of pathogenic *E. coli* from clinically healthy chickens and their

environment in poultry farms and hatcheries from the Lusaka and Chisamba districts of Zambia.

Materials and methods

Study site

The study was conducted in two districts of Zambia namely Lusaka and Chisamba (Fig. 1) from which three (3) commercial poultry farms A, B, C were randomly selected (Acharya et al., 2013). Each site has a hatchery and poultry breeder farm. In Lusaka, there were four hatcheries and four poultry breeder farms while in Chisamba, there were three hatcheries, and poultry breeder farms and three sites were randomly selected. The study took place between January 2018 and December 2018.

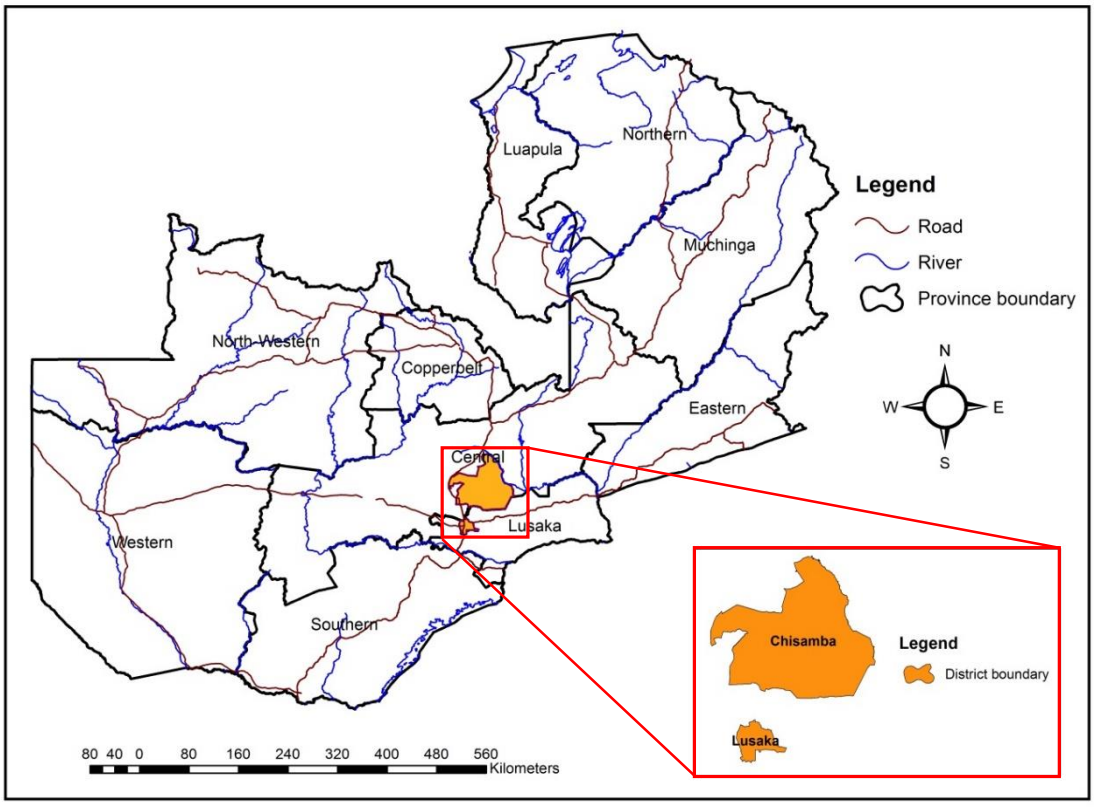

Fig. 1. Defining the study site of Lusaka and Chisamba districts of Zambia

Sampling design

A cross-sectional study design was conducted and in each study site eggs, environmental and cloacal swabs containing fecal matter were collected.

Sampling

From each poultry house, female adult birds were selected at random after obtaining verbal consent from the owners. The birds were restrained following the guidelines stipulated by the Animal Health Act 27 of 2010 of the country. Cloacal swabs, containing fecal matter were collected from selected laying and active or alert birds, which were not showing any clinical signs of infection (Karim et al., 2019). Environmental samples were collected from litter (about 10g) in poultry houses while floors, walls, and equipment of the hatchery rooms such as the setters and hatchers were sampled using sterile swabs, and eggs were collected from the poultry houses. The swabs were immediately put in the buffered peptone water (BPW) transport medium on transit to the Central Veterinary Research Institute (CVRI) laboratory in

Lusaka while maintaining the cold chain. The bacteriological culture process commenced immediately after arrival in the laboratory.

In the laboratory, eggs were cleaned with 70% alcohol before the collection of their contents using the sterile swab and put into the buffered peptone water.

Culture and biochemical identification

Each specimen was cultured on Mac Conkey agar (Merck, Germany) solid media and incubated at 37ºC overnight. Suspected colonies of *E. coli* identified by exhibiting dry, doughnut-shaped pink (Lactose fermenters) were sub-cultured on Eosin Methylene Blue (EMB) medium and incubated at 37ºC overnight. Colonies that showed metallic green color were suggestive of *E. coli*. Gram stain and microscopy were performed to confirm morphology and staining characteristics. The suspected colonies were then subcultured on the nutrient medium and incubated at 37ºC overnight. The isolates were subjected to biochemical procedures such as fermentation of glucose, utilization of citrate, Indole, adonitol, triple sugar iron, and decarboxylation of lysine (Blackburn and Mcclure, 2009) and incubated at 37ºC overnight.

Determination of the pathogenicity of E. coli isolates

Bacterial colonies identified as *E. coli* on culture and biochemical tests were further investigated by *in vitro* pathogenicity test using Congo red dye (CR test) binding activity as described by Berkhoff and Vinal and Ugwu et al. (Berkhoff and Vinal, 1986; Ugwu et al., 2020). Results of Congo red binding were recorded after 24 hours of incubation at 37ºC, followed by 48 to 72 hours of incubation at room temperature. Micro-organisms, which failed to bind the Congo red dye within 72 hours of incubation at room temperature and produced white colonies, were recorded as non-pathogenic *E. coli* while those exhibiting red colonies within 72 hours of incubation were recorded as pathogenic *E. coli* (Sharma et al., 2006; Hofstra and Veld, 1988). All the pathogenic strains were stored at -20°C in a mixture of skimmed milk, tryptose soy, glucose with 10% glycerol for antimicrobial susceptibility testing at a later stage.

Antimicrobial resistance and susceptibility testing

Kirby-Bauer diffusion susceptibility method (Hudzicki, 2009) was used to determine the antimicrobial susceptibility and resistant patterns of the isolated pathogenic *E. coli* strains The procedure was achieved by sub-culturing the organism on Mueller Hinton (MH) agar (Himedia, India) of about 4mm depth with standard antimicrobial diffusion discs placed on the surface of the agar and incubated at 37ºC for 18 to 24 hours. Pathogenic *E. coli* strains were tested against six (6) antibiotics which included; cephalexin 30mcg, co-trimoxazole 25mcg, (1.25 mg trimethoprim/23.75 mg sulfamethoxazole), gentamicin 10mcg, nalidixic acid 30mcg, tetracycline 30mcg, and streptomycin 30mcg (Himedia, India). The organism *E. coli* ATCC 25922 was included as the control to provide quality assurance. The diameter of the inhibition zone of growth was measured using a vernier caliper. The CLSI M100 ED 28-2018 was used for interpretation of the results (Replaces et al. 2018).

Data analysis

Data on prevalence and antimicrobial resistance (AMR) was analyzed using Microsoft Excel data analysis tool and Epi info™ 7.0.8.0 version 7.2.0.1, a computer statistical package from the Centre for Disease Control and Prevention (CDC, GA, USA).

Results

Detection of E. coli strains

A total of 417 samples were collected from the three farms as follows: farm A (n=221), farm B (n=80) and farm C (n=116) samples. Out of 417 samples, *E. coli* was isolated from 333 (79.9%) 95% CI=75.23-82.98% samples of which 62 (18.6%) 95% CI=14.90-23.28%, were pathogenic, while 271 (81.4%) 95% CI=76.72-85.1% were non-pathogenic (Table 1). The results showed that of the 62 pathogenic *E. coli*, farm A had 30 isolates, farm B had 25 isolates and farm C had 7 isolates (Table 1).

Antimicrobial susceptibility patterns of the E. coli isolates

The isolates were resistant to TET (100%), CEX (92%), COT (81%), NAL (42%), and STR (40%), while they were susceptible to GEN (77%) (Table 2).

Multi-drug resistant (MDR) of the 62 pathogenic *E. coli* isolates were determined and the results showed that three 3/62(4.8%) isolates were resistant to all the six antibiotic combinations, eleven isolates (17.7%) were resistant to five drugs, (33.9%) were resistant to four drugs, (22.6%) were resistant to three drugs, while (21.0%) were resistant to two drugs (Table 3). Henceforth, there were 49 isolates that potentially exhibited multi-drug resistance with different antibiotic permutations (Table 4).

Table 1. Prevalence of pathogenic *E. coli* from the three hatcheries and poultry farms

Sample type	FARM A (n=221)					FARM B (n=80)					FARM C (n=116)					Overall total n=417		
	Total	*E. coli* isolated		Pathogenic *E. coli*		Total	*E. coli* isolated		Pathogenic *E. coli*		Total	*E. coli* Isolated		Pathogenic *E. coli*		No. of samples	*E. coli* isolated	Pathogenic *E. coli*
		No.	%	No.	%		No.	%	No.	%		No.	%	No.	%			
Eggs	13	0	0	n/a	n/a	8	1	1.2	0	0	12	0	0	n/a	n/a	33	1(0.2%)	0(0%)
Litter	8	8	3.6	3	1.6	4	4	5.0	3	4.4	8	6	5.2	0	0	20	18(4.3%)	6(1.8%)
E/Swabs	0	0	n/a	n/a	n/a	7	1	1.2	0	0	0	n/a	n/a	n/a	n/a	7	1(0.2%)	0(0%)
C/swabs	200	169	76.5	27	15.3	61	62	77.5	22	32.4	96	82	70.7	7	8.0	357	313(75.1%)	56(16.9%)
Total	221	177	80.1	30	**16.9**	80	68	85	25	**36.8**	116	88	75.9	7	**8.0**	**417**	**333(79.9%)** 95%CI 75.2-83.0%	**62(18.6%)** 95% CI 14.9-23.3%

X^2= 154; p-value of *E. coli* is p=<0.01.There is a statistically significance

X^2= 3.1; p-value of the pathogenic *E. coli is p*= 0.37, indicating that there is no statistically significance

n/a=Not applicable

E/swabs= Environmental swabs

C/swabs= Cloacal swabs

Table 2. Susceptibility and resistance pattern of pathogenic *E. coli* according to individual antibiotic

Antibiotic	Resistant		Intermediate		Susceptible		Total
	Standard Inhibition zone	No. of samples	Standard Inhibition zone	No. of samples	Standard Inhibition zone	No. of samples	
Cephalexin 30mcg/dis	≤14	57 (92%)	15-22	0 (0%)	≥15	5 (8.1%)	62
Co-Trimoxazole 25mcg/dis	≤16	50 (81%)	11-15	2(3.2%)	≥10	10 (16.1%)	62
Gentamicin 10mcg/dis	≤12	5 (8.1%)	13-14	9(15%)	≥15	48 (77.4%)	62
Nalidixic Acid 30mcg/dis	≤13	26 (42%)	14-18	24(39%)	≥19	12(19.4%)	62
Tetracycline 30mcg/dis	≤14	62 (100%)	12-14	0	≥15	0	62
Streptomycin 30mcg/dis	≤14	25 (40.3%)	12-14	32(52%)	≥15	5(8.1%)	62
	p-value=<0.01		p-value=<0.01		p-value=<0.01		

Mcg = Micrograms

Discussion

The isolation of 79.9% *E. coli* suggests that the bacteria were distributed both in the animals and the environment. In the current study, 75.1% of isolates were from the cloacal of the birds, followed by 4.3% from the litter of the poultry house while 0.2% isolates were from the eggs and surface of the hatchery rooms respectively. This distribution of the bacteria suggests that the bacteria were in every aspect of the environment.

Isolation of the pathogenic *E. coli* was mainly in the cloacal of the birds. Our findings suggest that pathogenic *E. coli* could easily be transmitted into the community via birds and their products. These findings are consistent with those reported by Belanger et al and Mainda. (Belanger et al., 2011; Mainda, 2016). The isolation of pathogenic *E. coli* from the poultry houses and hatchers is an indication of inadequate biosecurity at the poultry houses. This situation has the potential to cause great concern to the poultry farmers and those who purchase the day-old chicks from these poultry breeders (Van de Bogaard et al., 2001). It also suggests that workers may be at high risk of being infected with the bacteria. The management at these farms needs to enhance infection prevention measures and strictly observe biosecurity measures. It has been reported elsewhere that farmworkers, poultry, and its product can spread the infection to the community (Van de Bogaard et al., 2001). Poultry meat has been recognized as a cheaper source of protein, hence increasing the demand. On the other hand, there have been concerns of poultry meat being carriers of food-borne diseases, which could transmit antimicrobial resistance genes to humans (Woolhouse et al., 2015). In this study, 18.6% of pathogenic *E. coli* isolates had a slightly lower isolation rate as compared to 25% isolation in another related study in Zambia (Munang'andu et al., 2012).

Table 3. Frequency of multi-drug resistance among the pathogenic *E. coli* isolates

No. of antibiotics	No. of Isolates resistant	Percent	Chi-square, p-value
1	0	0%	
2	13	21.0%	X^2=14;
3	14	22.6%	p=0.01
4	21	33.9%	
5	11	17.7%	
6	3	4.8%	
Total	**62**	**100.0%**	

The result is significant at $p < 0.05$.

Table 4. The frequency of antibiotic resistance profile of isolates of pathogenic *E. coli*

Permutation of antibiotics	No. of isolates	Percentage	Chi-square, p-value
CEX+TET	9	14.5	
CEX+NAL+TET	1	1.60	
CEX+COT+GEN+TET	10	16.1	
CEX+COT+TET+STR	9	14.5	X^2=44
CEX+COT+NAL+TET+STR	10	16.1	p=<0.01
CEX+COT+GEN+NAL+TET+STR	3	4.80	
CEX+COT+GEN+NAL+TET	1	1.60	
CEX+NAL+TET+STR	1	1.60	
COT+NAL+TET+STR	1	1.60	
CEX+COT+TET	12	19.4	
CEX+GEN+TET	1	1.60	
COT+TET	4	6.50	
TOTAL	**62**	**100**	

The result is significant at $p < 0.05$.

KEY

CEX= Cephalexin, TET= Tetracycline; NAL= Nalidixic Acid; COT= Co-Trimoxazole; GEN= Gentamicin; STR= Streptomycin

Other studies have shown a slightly higher prevalence of pathogenic *E. coli* probably due to different approaches in the two studies, for example, Munang'andu et al. 2012, focused on the different types of bacterial isolates, while this study focused on the isolation of *E. coli*

The high AMR results suggest that livestock has become a focal point of spreading AMR based primarily on the number of antimicrobial agents used in food and animal production (Schmidt et al., 2014). Antimicrobial resistance (AMR) has been recognized as an emerging problem worldwide both in human and veterinary medicine. Our study showed that pathogenic *E. coli* isolated were 100% resistant to tetracycline drug. These results are consistent with those previously described (Sharma et al., 2016; Filho et al., 2015). Other reports suggest that the bacteria are most likely to contain tetracycline resistance gene (*Tet* genes), which could have been responsible for the absolute resistance (Zibandeh et al., 2016). The whole concept is an indication that the bacterium has been exposed previously to the antibiotic at an inappropriate dose either during treatment or prevention. Besides, the bacteria might have acquired some resistant genes from the environment. Nevertheless, it has been reported elsewhere that tetracyclines, which make up another 40% of total antimicrobials used in animal production, are not considered a first-line antimicrobial for treatment in human medicine (Rasheed et al., 2014). However, there are several antimicrobials administered to animal foods that are analogues to human therapeutic compounds. Many studies have reported resistance to antimicrobials that are critical in fighting human disease from food, animal, and environments. The diverse food and environmental harbors micro-organism, especially bacteria, that are resistant to one or more antimicrobial drugs (Economou et al., 2015). *E. coli* bacterium is responsible for urinary tract infections (UTIs), a common cause of both community and nosocomial infections in patients admitted to nursing homes and hospitals (Ron, 2006). In addition, resistant *E. coli* strains can transfer antibiotic resistance genes or traits not only to other *E. coli* strains but also to other bacteria within the gastrointestinal tract (Ginns et al., 1996). The result in this study suggests the possibility of the spread of antibiotic-resistant *E. coli* from animals via poultry and its products. Furthermore, an increase in tetracycline drug use in human primary healthcare medicine, the clonal spread of resistant commensal bacteria, and an increase in resistant *E. coli* pathogens, have been reported (Van de Bogaard et al., 2001). The finding is consistent with studies elsewhere which suggested that there is a significant increase in the incidence of antimicrobial resistance (AMR) in the *E. coli* strains in chickens (Nhung and Carrique-Mas, 2017). The drug resistance was probably due to increased misuse or abuse of antibiotics as feed additives for growth promotion and prevention of diseases. Others may include the use of inappropriate antibiotics for the treatment of diseases, resistance gene transfer among different bacteria, and possible cross-resistance between antibiotics used in the poultry industry (Mellata, 2013).

Cephalexin (92%) and tetracycline drugs were used to treat recurrent cystitis infections caused by *E. coli*. Its resistance reduces the drug treatment options in sick birds infected with resistant *E. coli* strains. Resistance to co-trimoxazole was observed at (81%) and its extensive use could be a contributor to resistance in poultry. This study is consistent with Mshana et al. who reported an increasing trend of resistance to commonly used antibiotics such as tetracycline, co-trimoxazole, gentamicin, erythromycin, ampicillin, and third-generation cephalosporin (Mshana et al., 2013). In Zambia, livestock farmers purchase the antibiotics upon production of a prescription form from a registered veterinarian. However, these drugs might be sold without prescription if the farmer has shown proof that the livestock was sick by responding correctly to the pharmacist interrogations. It is a common practice when there

is no veterinarian nearby to prescribe the antibiotic, especially, livestock farmers in the remote areas of the country (Kalungia et al., 2016).

Our study showed that 3/62(4.8%) isolates were resistant to all the six antibiotics combinations, 11/62(17.7%) were resistant to five drugs, 21/62(33.8%) were resistant to four drugs. Isolates were classified as multi-drug resistant (MDR) if the bacteria were resistant to ≥ 3 classes based on susceptibility to more drug combinations. Multiple antibiotic resistance may probably be acquired through mobile genetic elements such as plasmids, transposons, and Class 1 integrons or from the acquisition of genes encoding efflux pumps (Talebiyan et al., 2014; Markey et al., 2013). The multi-drug resistant isolates offer narrow treatment options in chicken farming which provides an increase in the risk of incidence of human infections and complicating their treatment. In this aspect, the study is in agreement with results reported by Talebiyan et al., who suggested that chicken carried multidrug-resistant *E. coli* strains with high-level resistance to oxytetracycline, chlortetracycline, and sulfadimethoxine-trimethoprim (Talebiyan et al., 2014). Some bacteria may acquire antimicrobial resistance by the possession of extended-spectrum beta-lactamases (ESBL) enzymes. This phenomenon has been reported in the ESBL- producing *E. coli* collected from different localities (Hassan, 2014). Rashid et al. reported that extended spectrum beta-lactamases (ESBL) are encoded by gene traits located on large plasmids, and these also carry genes for resistance to other antimicrobial agents (Rashid, 2015).

Other animals may obtain antimicrobial-resistant *E. coli* or resistant genes to the antibiotics directly through contact with animals, food of animal origin, or the environment (Hammerum and Heuer 2009; Skurnik et al., 2015). Avian strains of *E. coli* are potentially zoonotic pathogens and have the potential to infect humans. Poultry (both domestic and wild birds) can act as a reservoir for virulence genes for *E. coli* to human beings (Smith et al., 2007; Blaak et al., 2015). This study also revealed high sensitivity of the isolates to gentamicin (77%) which was similar to those that showed antimicrobial sensitivity (AMS) of the pathogenic *E. coli* isolated (Shobrak and Abo-Amer, 2014). The study revealed that the bacteria were less resistant to gentamicin drug and could be used as an alternative drug to control *E. coli* infections. The findings suggest that antibiotics such as tetracycline might have been abused on the farms leading to the development of resistance of *E. coli* bacteria. The use of antibiotics in livestock settings as growth promoters or as nonspecific to treat and prevent infection has probably attributed to antibiotic consumption and builds up resistance among bacteria.

Conclusion and recommendations

It's evident that pathogenic *E. coli* strains isolated from the cloacal swabs and litter were 100% resistant to tetracycline, 92% to cephalexin, and 81% to co-trimoxazole. It also suggests that 4.8% of bacterium exhibited MDR to all the six antimicrobial agents used in the study. Therefore, it is desirably important to observe biosecurity measures and good-hygienic practices at the farms to prevent contamination with infected poultry and its products. Veterinary officers should strengthen education campaigns on biosecurity measures and good-hygienic practices, to prevent contamination at the farm and the community. This can be achieved through workshops and seminars of the farmworkers and community members.

Acknowledgements

The authors would like to thank the members of staff at Central Veterinary Research Institute especially in the bacteriology section for the assistance rendered to them. They would like to thank the Director of Veterinary Services for allowing the collection and analysis of samples. We would like to thank Mr. Milner Mukumbwali for assistance in the mapping of the study site.

Conflict of interest statement

There is no conflict of interest.

Financial disclosure

Not applicable

Ethical approval

Ethical approval to conduct this study was sought from the Directorate of the Department of Veterinary Services. Verbal consent to participate in the study was granted by the poultry farmers where samples were collected and analyzed.

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