Antibiotic Resistance Pattern among *Escherichia Coli* Isolated From Poultry Farms.

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Abstract

The aim of this study was to isolate and characterize *E. coli* from broilers chickens in Bahri locality in Khartoum state and to elucidate their antimicrobial resistance profile. A total of 100 random cloacal and fecal samples were collected in the period of July to September 2018 from broilers farms in Bahri locality. The biochemical method identified 50 samples at E. coli. These 50 samples were further subjected to polymerase chain reaction (PCR) for the 16SrRNA gene that identified 20 isolates at E. coli. Concerning the antibiotic resistance profile, 16 isolates out of the 20 isolates demonstrated resistance to Erythromycin and Clarithromycin with resistance percentages of (80%) for each. Also only 3 isolates demonstrated susceptibility to Azithromycin (15%) while 17 isolates demonstrated resistance to Azithromycin (85%). In general the isolates demonstrated resistance to macrolides with resistance percentage ranges between 80 to 85%. In case of the Tetracycline, the isolates also showed resistance percentage of (80%). The isolates showed moderate resistance to the Ciprofloxacin since 9 isolates showed susceptibility to the Ciprofloxacin (55% resistance). It is noteworthy that according to the number of isolates the prevalence of *E. coli* in this study was found to be 20%. Taken together, distinct from the other studies conducted in Khartoum State, this study used the molecular characterization method (PCR) for identification of the isolates as *E. coli* which is considered as an accurate and sensitive mean. Moreover the isolates exhibited antibiotic resistance patterns to the tested antibiotics which may raise the imprudent use of antibiotics in broilers industry.

Keywords: *E. coli*, Tetracycline, Azithromycin, Ciprofloxacin, Antibiotics.

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Introduction

Escherichia coli have been considered as one of the most important foodborne pathogens all around the world. The genus of *E. coli* is a gram-negative bacterium, rod-shaped, and classified as a member of the family Enterobacteriaceae within the Gammaproteobacteria class [1]. *E.coli* has been associated with a variety of diseases in poultry such as perihepatitis, pericarditis, airsacculitis, salpingitis, peritonitis, panophthalmitis, omphalitis, colispticemia, coligranuloma, cellulitis and swollen-head syndrome [2].

Strains of *E. coli* predominate among the aerobic commensal flora in the gut of humans and animals. These bacteria are present and widespread wherever there is fecal contamination, causing pollution of water sources, drinking water and food [3]. The species encompasses a variety of strains, which may be purely commensal or possess combinations of pathogenic mechanisms that enable them to cause disease in man and other animals [3].

Transmission and mode of the infection the primary and secondary habitats of *Escherichia coli* are the intestinal tract of warm-blooded animals and the environment, respectively. Although many *E. coli* strains are harmless commensals, a subset has acquired the ability to cause intestinal or extraintestinal diseases. Based on the host and the site of infection, different *E. coli* strains are subclassified as neonatal meningitis *E. coli* (NMEC), sepsis-associated *E. coli* (SEPEC), uropathogenic *E. coli* (UPEC), which cause newborn meningitis, sepsis, and urinary tract infections (UTI), respectively, and avian pathogenic *E. coli* (APEC), which mainly causes respiratory and systemic disease in poultry [4]. Among the diseases some are often severe and sometimes lethal infections such as meningitis, endocarditis, urinary tract infection, septicemia, epidemic diarrhea of adults and children [5]. *E. coli* infections in birds cause many clinical manifestations which are characterized by respiratory diseases that are frequently followed by generalized infections which end by death [2].

E. coli infections are important to human health and are a major cause of economic loss to the poultry industry. It can colonize the intestine, similar to non-pathogenic commensal *E. coli* but are equipped with virulence factors that allow them to cause disease in extraintestinal sites.

In addition to the intestine, poultry houses serve as a reservoir for *E. coli*, and this environment allows strains to persist for many months over successive flocks [4]. Chicken-to-chicken *E. coli* transmission, through pecking or inhalation of contaminated fecal dust, could result in carcass condemnation and severe disease or death of poultry [6]. *E. coli* transmission among chickens may increase the presence of *E. coli* colonized chickens, and thus increase the frequency of *E. coli* transmission onto poultry products. It includes not only commensal strains but also pathogenic ones that cause a variety of human diseases—resulting in more than 2 million deaths each year [1]. *E. coli* strains are classified by virulence properties and pathogenicity mechanisms causing gastrointestinal diseases such as diarrhea.

Signs of *E. coli* in poultry since *E. coli* are an opportunistic pathogen and attack a number of organs, infections can cause a wide variety of signs or symptoms. Symptoms may range from sudden death of the bird to a vague sense that the bird is not doing well [2]. Symptoms also depend on the age and general health of the bird. Generally, birds will appear unthrifty and have ruffled feathers. They may also be depressed and have a decreased appetite. During the acute phase of the disease, you may also notice yellowish colored droppings and birds may be soiled in the vent region [2]. The main clinical signs of naturally infected chicks with E.coli were reported as depression, loss of appetite, tendency to huddle respiratory distress, reduction of weight gain, dropped wing, closed eyes, cyanosis and labored breathing [3].

E. coli Treatment and Preventionis still an emerging field in colibacillosis studies. Most treatments do not focus on already occurring colibacillosis infections, rather in the prevention of the disease. Treatments include antibiotics and attempts to control the resulting infections. Growing concern about antibiotic resistance has also affected the way colibacillosis is being treated [4]. This increasing rate of resistance poses a real threat to clinicians and farmers around the world [5].

Multi antibiotic resistance index (MAR)

Antibiotic resistance to more than 3 antibiotics termed multi antibiotic resistance index (MAR) of the isolates was investigated followed modified method of [6].

Materials and Methods

Collections of specimens

A total of 100 fecal and cloacal swabs were collected from close and semi-close poultry broilers farms. Sterile cotton swabs were inserted deeply in the cloaca and then rotated 3 to 5 times then pulled out gentle and placed in a sterile container. Fresh fecal samples were collected from the floor from different sites within the farm by a gloved hand.

Biochemical identification of the isolates

For isolation and identification of *Escherichia coli* from the collected samples, the samples were first enriched by incubation for 12 hours at 37°C, sub-culturing on nutrient agar plates to purify colonies followed by incubation at 37°C for another 24 hours. Purified isolates were further identified according to the reaction of Gram's stain, shape of the bacterial colonies, motility, colonial characteristics on selective media such as Eosine Methylene Blue agar media (EMB).The biochemical tests were performed according to the methods detailed in Cowan and Steel's Manual for the Identification of Medical Bacteria [7]. Tests performed included, Catalase-Oxidase tests, Indole test, Citrate utilization, Motility test, Kligler test, Voges- Proskauer reaction test (VP), Methyl red (MR) test and Indole test

Molecular characterization

The molecular characterization of the isolates was also performed for the confirmation of the isolates as *E. coli* using Polymerase Chain Reaction (PCR). The target gene for the identification of the isolates was 16SrRNA gene.

DNA extraction: Samples that were retrieved from the conventional methods and suspected as *E. coli* spp were further analyzed by molecular methods using Polymerase Chain Reaction, PCR. The DNA was extracted from the sample according to the method described by [6].

Primers: The primers used for the PCR amplification were previously described by [5]. The forward primer was F-5'-GGGAGTAAAGTTAATACCTTTGCTC-3' and the reverse primer was R- 5'-TTCCCGAAGGCACCAATC-3'. The primers were donated by the laboratory of Molecular Biology and Bioinformatics at the University of Bahri.

PCR amplification: The components of the reaction mixture were optimized as follows: 2 µl from extracted DNA as a template, 1µl from the forward, 1µl reverse primers. These components were added to ready master mix containing loading dye and the final volume was completed to 25 µl with DW water. PCR reaction was performed in gene Amp PCR system (England) with a heated lid. The PCR reaction conditions were stated as previously described [2]. The PCR reaction conditions were as followed: 94˚C for 5 minutes (initial denaturation) and 94˚C for 2 minutes (denaturation). The annealing temperature was 56˚C for 45 seconds. The extension was set to 72˚c for 1 minute, followed by 72˚C for 10 minutes as a final extension. The number of the PCR cycles was set to be 35 cycles.

DNA gel electrophoresis: DNA electrophoresis 50X stock solution was prepared as follows: 242 g tris, 37.2 g Na2 EDTA, 800 ml distilled water and was added and mixed thoroughly. A volume of 57.1 ml of the acetic acid was added and the final volume was completed to one liter with deionized water. For

the DNA electrophoresis running solution: 1X working solution: dilute the 50X to 1X by distilled water.

Antibiotic sensitivity test

Minimum inhibitory concentration (MIC) of the isolated strains was determined by using the broth microdilution method recommended by the clinical laboratory standards institute [6]. The antimicrobials tested consisted of the following macrolides: Erythromycin (ERY), Azithromycin (AZM) and Clarithromycin (CLA). The florouquilones Ciprofloxacin (CIP) and the Tetracycline (TET) were also tested. Resistant breakpoints were as following: ERY≥32µg/ml, AZM≥8µg/ml, CLA≥16µg/ml, CIP≥8µg/ml, and TET≥16µg/ml [7].

Results

Biochemical examination

The summary of the results of all biochemical tests used in this study were summarized in (Table 1) (Figure 1).

Characteristics	E. coli
Gram's stain	-ve bacilli
Motility	Motile
Colony shape	Flat 2-3 mm
Colonial characteristics	2-3 mm flat, metallic shining in Eosin Methylin
	Blue, Lactose fermenter in McConkey
Citrate utilization	-ve
Voges- proskauer (VP) reaction	+ ve
Kligler test	- ve
Methyl red test	+ ve
Indole test	+ ve
Growth in EMB media	metallic shining

Table 1. *Biochemical characteristics used for identification of the isolates E. coli (+ ve) positive (- ve) negative.*

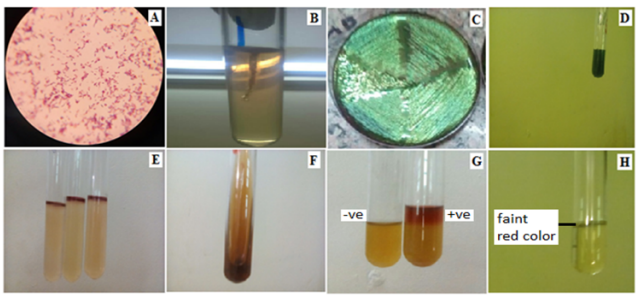

Figure 1. *(A) Grams stain, (B) Motility, (C) The growth of E. coli on agar media, (D) Citrate utilization, (E) Voges-proskauer, (F) Kilgler test, (G) Methyl red test and (H) Indole test.*

The thin smears were prepared from colonies collected from MacConky and Nutrient agar for Gram's staining. As shown in figure (1) the staining revealed the isolates as Gram–negative, pink colored, small rod shaped appearance, arranged in single or paired under the microscopic examination. The motility test was performed using semi-solid media (stabbing method). Figure (1) showed that the bacteria were obviously moved within the semisolid media. Moreover bacterial colonies in the EMB media with metallic shining characteristics may indicate the growth as E. coli. Figure (1) demonstrated that the bacteria colonies appeared as metallic shining colonies. Moreover citrate utilization test for was shown to be negative since no growth on the slant and no change in color to blue of the medium was observed. This indicated a negative result. Also the VP test provided that the isolates were positive for VP test as a pink color was observed at the top of the test. These results indicated the isolates as E coli. The Kligler test was shown to be negative since no change in the color to dark blue was observed. This negative result further indicated the isolates as E. coli. Methyl red (MR) test provided red coloration was obtained. This result indicated the test was positive for methyl red. This result indicated the isolates as E. coli. The isolates were found to be indole positive since a faint red ring was obtained. All these examinations tentatively indicted the isolates *E. coli* (Figure 2).

Figure 2. *The DNA was extracted and the quality of the DNA was determined in the gel electrophoresis.*

Molecular characterization

DNA extraction: As shown in figure (2) the quality of the DNA was checked in the gel electrophoresis. The Genomic DNA was shown as bands just passed out the wells of the gel after 30 minutes of run in the electrophoresis.

PCR amplification: The PCR was performed for the isolates that were obtained from the biochemical identification. Using the molecular identification method, 20 bacterial isolates were identified as E. coli. Demonstrated a band size (584 bp) that indicated the isolates as *E. coli* (Figure 3).

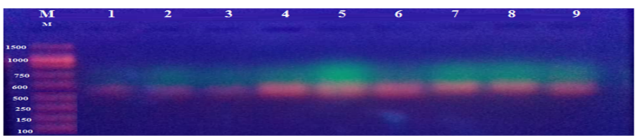

Figure 3. *The PCR amplification of 16SrRNA gene of the suspected isolates of E.coli. the band size was expected to be 584 bp that indicated isolates as E. coli. M was DNA ladder; lane 1, 2, 3, 4, 5, 6, 7, 8 and 9 were the amplicon of 16SrRNA gene that indicated isolates as E.coli spp.*

Sensitivity to antibiotics: Antibiotic sensitivity tested was performed according to CLSI, 2012 (Table 2).

Antimicrobial	ERY	CLA	AZM	TET	CIP
ERY	-	-	-	-	-
CLA	P=0.780	-	-	-	-
AZM	P=0.028 (OR=15)	P=0.028 (OR=15)	-	-	-
TET	P=0.364	P=0.330	P=0.414	-	-
CIP	P=0.435	P=0.134	P=0.348	P=0.671	-

Table 2. *Two way antimicrobial combination P and OR against E. coli isolates*.

Table 2 demonstrated the sensitivity test of *E. coli* isolates against five antibiotics commonly used for the treatment of *E. coli* infection in human and broilers. For the macrolides, Erythromycin Clarithromycin and Azithromycin the resistant breakpoints were ≥32µg/ml ≥16µg/ml and ≥8µg/ml, respectively. Out of the 20 tested isolates 16 isolates demonstrated resistance to each of Erythromycin and Clarithromycin (80%). Only three isolates out of the 20 isolates demonstrated susceptibility to Azithromycin (15%) while 17 isolates demonstrated resistance to Azithromycin (85%). In general the isolates demonstrated resistance to macrolides with resistance percentage ranges between 80 to 85%. In case of the Tetracycline the resistance breakpoint was ≥16µg/ml. The isolates also showed resistance to Tetracycline (80%). The isolates showed moderate resistance to the Ciprofloxacin. Out of the 20 isolates 9 isolates showed susceptibility to the Ciprofloxacin (55% resistance).

The MIC of the twenty isolates of E.coli against different antimicrobial agents, the MIC was determined by the broth microdilution method according to CLSI, 2012. (ERY: Erythromycin; CLA: Clarithromycin; AZM: Azithromycin; TET: Tetracycline; CIP: ciprofloxacin). Resistant breakpoints were as following: ERY≥32µg/ml, CLA≥16µg/ml, AZM≥8µg/ml, TET≥16µg/mland CIP≥8µg/ml.

As shown in figure (3) the average and the standard deviation (using the student t-test) of the sensitivity of the 20 isolates to each antibiotic was measure. The isolates showed least susceptibility to Erythromycin followed by Tetracycline, Clarithromycin. In case of the Azithromycin, beside the three susceptible isolates there were 8 isolates showed moderate resistance. Therefore the average of MIC for the 20 isolates showed low resistance. Isolates provided the best sensitivity to Ciprofloxacin as it scored the lowest MIC. Taken together the isolates showed resistance to Tetracycline and macrolides with exception of Azithromycin. In addition to that the isolates demonstrated the least resistance resistances to Ciprofloxacin.

The probability of the two way antimicrobial combination against *E. coli* isolates and the odd ratio (OR<1.0) was shown in (Table 2).

Discussion

Escherichia coli are one of the common microbial floras of gastrointestinal tract of poultry, human and other animals. This bacterium may become pathogenic to both poultry and human [1, 2].

Antibiotics are used in the poultry farms for multiple purposes such as growth promoters, prophylaxis and for therapeutic purposes [2,3]. These veterinary drugs include a large number of different types of compounds that can be administrated in the feed or in the drinking water. However the imprudent use of these drugs may exert adverse effects due to the presence of antibiotics residues and the presence of antibiotics resistant bacteria such as E. coli. Moreover there are multiple scientific evidences that demonstrated the relationship between the use of antibiotics in food producing animals and the emergence and selection of antibiotics resistance bacteria. Recent studies in Sudan and worldwide have reported antimicrobial residues and antibiotic resistant bacteria in food animal products such as chicken meat suggesting large-scale unregulated use of antibiotics by the poultry industry. This is consistent with our observations as we also found a marked predominance of antibiotic resistance among *E. coli* isolates obtained from fecal and cloacal swaps in broilers chicken. Moreover *E. coli* isolates in different studies were tested for antimicrobial resistance of multiple antibiotics with different testing procedures.

These isolates were grouped as susceptible, intermediate, resistant and multidrug resistant multidrug resistance was defined as resistance to three or more classes of antibiotics. In this study antimicrobial resistance was performed using broth microdilution method recommended for Erythromycin, Clarithromycin, Azithromycin, Tetracycline and Ciprofloxacin. In accordance to the antibiotics resistance breakpoints our results coincided with previous studies since the isolated *E. coli* provided resistance to all tested antibiotics with exception to Ciprofloxacin that showed moderate resistance. In addition to that our isolated *E. coli* demonstrated resistance to more the three antibiotics and thus showed multidrug resistance to the tested antibiotics. The isolates showed resistance to Tetracycline, Clarithromycin and Erythromycin with exception of Azithromycin. In addition to that, the isolates demonstrated the least resistance to Ciprofloxacin. For instance 16 isolates out of the 20 isolates demonstrated resistance to Erythromycin and Clarithromycin with resistance percentages of 80% for each. Also only 3 isolates demonstrated susceptibility to Azithromycin (15%) while 17 isolates demonstrated resistance to Azithromycin (85%). In general the isolates demonstrated resistance to the tested macrolides with resistance percentage ranges between 80 to 85%. In case of the Tetracycline, the isolates also showed resistance percentage of 80%. The isolates showed moderate resistance to the Ciprofloxacin since 9 isolates showed susceptibility to the Ciprofloxacin (55% resistance).This resistance in the *E. coli* isolates mainly attributed to the imprudent usage of the antibiotics in the broilers farms taken together this study demonstrated that *E. coli* is a major contaminant in broilers farms and maybe responsible for disease conditions in broiler A significant association was found between AZM with both ERY and CLA with OR=15. This indicates that when E.coli insolent is resistant to AZM it is 15 times more likely to be observed

resistant to ERY and CLA than when the insolent is sensitive. Multiple drug resistance might be due to the presence of transferable resistance plasmids.

Conclusion

This study confirmed the presence of susceptible and antibiotic resistant *E. coli* in Bahri locality in both semi close and closed system from poultry production farms. This study suggested that farms management practices play an important role in the level of the *E. coli* prevalence and may be the antibiotic resistance of the selected bacterial species with in differing poultry production farms. In this study twenty identified isolates were found resistant to at least one antibiotic which has raised some concerns about the efficacy of poultry antimicrobial therapy. The molecular method confirmed the results of traditional detection method of *E. coli* species with high specificity and sensitivity as potentially valuable tool of the detection method.

Conflict of Interest

The authors declared no competing interest.

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