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Research Article

Identification and Antibiotic Resistance Patterns of *Escherichia coli* Isolated from Broilers Farms in Bahri Locality/Sudan

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Abstract

The aim of this study was to isolate and characterize *Escherichia 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*. This 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*; Broiler Farms; Biochemical Identification; PCR; Antibiotic Resistance

Introduction

Poultry has been on earth for over 150 million years, dating back to the original wild jungle fowl. Ducks, geese, turkeys, pigeons, and chickens are on the list of the species under the general term poultry [1]. In Sudan, poultry products were commenced on a commercial basis in 1979 by Sudanese Kuwaiti poultry production Company. As a commodity, poultry products are highly demanded by the locals and the public of the neighboring countries especially Arabs [2].

Poultry meat is an important food product and constitutes a substantial portion of daily-consumed proteins. It is the second most widely eaten type of meat in the world, i.e. chicken, turkey, duck, geese, domesticated quail [3]. Poultry meat and eggs are preferred rather than the other kinds of animal food products for a variety of reasons, dietary poultry meat is easy to prepare at home- and widely used in restaurants and fast – food establishment [4,5].

Poultry keeping is an old practice, where the domestic fowl had been kept for generations in villages and backyards of dwellings to supply both eggs and meat for consumption. In the late seventies, with an increase in demand for poultry products, many investors from the Arab Gulf States started commercial egg and broiler production in Sudan. Ever since, the poultry industry significantly increased. This resulted in distribution of commercial poultry farming in Khartoum state and a round other big cities. With this growing industry, farmers in Sudan switched from open to the closed and semi-closed system [6]. The rapid expansion of poultry

production in Sudan in recent years has stimulated many workers to study poultry major diseases that result in severe economic losses [7].

Colibacillosis has an important economic impact on poultry production worldwide. The majority of economic losses resulted from mortality and a decrease in productivity of the affected birds. It is a common disease in poultry flock especially in the intensive farming system [8]. Signs in birds affected with collibacillosis vary from sudden death to birds being off-color with their necks pulled into their bodies [9].

Several techniques for detection of *E. coli* spp. in fecal material are used such as cultures with selective media followed by a series of biochemical test. Identification of *E. coli* spp can also be performed by molecular methods such as PCR. This procedure is the most important development for research in molecular biology and it is fast, as well as highly sensitive and very specific [10]. The antibiotic resistance in poultry is now generally known to be due to the widespread use of antibiotics. This is considered as the main risk factor for the increase in the occurrence of antibiotic-resistant bacterial strains [11,12]. The aim of this study was to isolate and assess the antibiotic resistance profile of *E. coli* isolated from broilers farms in Bahri locality in Sudan.

Material 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. Each 10 fecal samples were placed in one sterile bag as one sample.

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 [13]. 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 [14].

Primers

The primers used for the PCR amplification were previously described by [15]. 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 [15]. 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 [16]. 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 [16].

Results

Biochemical examination

The summary of the results of all biochemical tests used in this study were summarized in table 1 and figure 1. The thin smears were prepared from colonies collected from Mc Conky 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 semisolid 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*.

Characteristics	E. coli
Gram's stain	-ve bacilli
Motility	Motile
Colony shape	Flat 2-3 mm
Colonial Characteristics	Flat 2-3 mm, metallic shining in Eosin Methylin Blue, Lactose fermenter in MacConkey
Citrate utilization	-ve
Voges-proskauer (vp) reaction	+ve
Kliger test	ve-
Methyl red test	ve+
Indol test	ve+
Growth in EMB media	Metallic shining

Table 1: biochemical characteristics used for identification of the isolates *E. coli.*

(+ ve) Positive (- ve) Negative

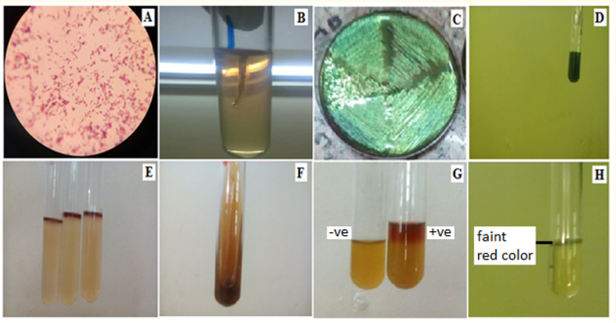


Figure 1: (A) Gram's stain, (B) Motility, (C) the growth of *E. coli* on agar media, (D) Citrate Utilization, (E) Voges- Proskauer, (F) kilgler test, (G) Methyl red test and (H) Indole test.

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.

Figure 2: The DNA was extracted and the quality of the DNA was determined in the gel 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*. Figure 3 demonstrated a band size (584 bp) that indicated the isolates as *E. coli*.

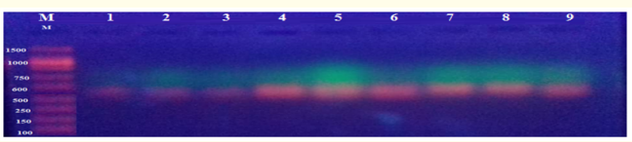
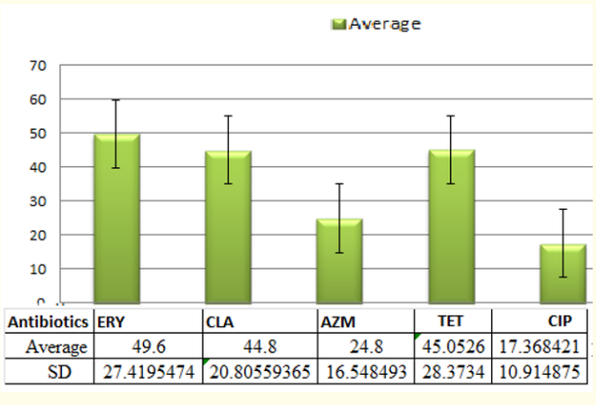


Figure 3: The PCR amplification of 16SrRNA gene of the suspected isolates of *E.coli*. The band size was expected to be 584 bp that indicated isolates as *E. coli*. M was DNA ladder; lane 1, 2, 3, 4, 5, 6, 7, 8 and 9 were the amplicon of 16SrRNA gene that indicated isolates as *E. coli* spp.

Sensitivity to antibiotics

Antibiotic sensitivity tested was performed according to CLSI, 2012. Table 2 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).

As shown in figure 4 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.



Antibiotics	ERY	CLA	AZM	TET	CIP
Average	49.6	44.8	24.8	45.0526	17.368421
SD	27.4195474	20.80559365	16.548493	28.3734	10.914875

Figure 4: The average and the standard deviation (SD) of the twenty isolates MIC to each antibiotic used using the student t test.

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 [17,18].

MIC ug/ml					
Isolate	**ERY**	**CLA**	**AZM**	**TET**	**CIP**
1	16	32	16	-	16
2	64	32	32	64	32
3	64	64	16	32	8
4	32	64	32	64	32
5	64	32	16	16	8
6	16	16	8	32	8
7	32	16	16	32	8
8	64	64	32	64	16
9	64	64	32	64	16
10	32	64	16	64	8
11	64	64	16	128	32
12	16	32	16	8	16
13	64	64	32	32	8
14	64	32	64	16	16
15	64	64	16	16	8
16	32	16	4	32	2
17	16	32	4	64	32
18	64	64	32	32	-
19	128	64	32	64	32
20	32	16	64	32	32

Table 2: 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, AZM≥8µg/ml, CLA≥16µg/ml, CIP≥8µg/ml, and TET≥16µg/ml (CLSI, 2012).(-): not assessed.

Although most isolates of *E. coli* are nonpathogenic but they are considered as indicator of faecal contamination in food and about 10 to 15% of intestinal coliforms are opportunistic and pathogenic serotypes [19] and cause a variety of lesions in immunocompromised hosts as well as in poultry. 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 [20]. Therefore it is of great importance to study *E. coli* in broilers and to assess the antimicrobial resistance profile of the isolates to the commonly used antibiotics.

Generally the bacteriological conventional methods were commonly used for detection and identification of bacterial organisms as a preliminary identification of bacterial organisms. However the application of PCR for detection and identification of bacterial organisms has been described by several workers [21,22]. In this study 100 fecal and cloacal samples were collected from broilers farms in Bahri locality. The bacteriological identification methods identified 50 isolates as *E. coli*. However the described PCR assay in this study reproducibly and specifically detected only 20 isolates as *E. coli* from fecal and cloacal sample with specific 584 bp PCR products of the 16S rRNA gene. Therefore the sensitivity of the PCR was comparable or even more sensitive than the conventional bacteriological procedure for specific identification of organisms. According to this confirmed number of the isolates as *E. coli* the prevalence of *E. coli* in this study was found to be 20%. This figure was nearly lower than the previous report of Mohamed-Noor., *et al*. [23] who reported a prevalence of 44%. However the prevalence in this study was higher than that reported by Abdulahi., *et al*. [24] who reported a prevalence of 8.9% in closed system farms than open system (3.2%) in broilers farms in Sudan.

Antibiotics are used in the poultry farms for multiple purposes such as growth promoters, prophylaxis and for therapeutic purposes [23,25]. 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* [25,26]. 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 [23,25,26]. 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 [23,27,28]. 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 [29,30]. In this study antimicrobial resistance was performed using broth microdilution method recommended by CLSI 2012 for Erythromycin, Clarithromycin, Azithromycin, Tetracycline and Ciprofloxacin. In accordance to the antibiotics resistance breakpoints [16] 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 [23,27-31]. 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. Some studies demonstrated the resistance and virulence genes from avian pathogenic *Escherichia coli* (APEC) from broiler chickens [31,32]. Therefore further work is required to demonstrate these genes from avian pathogenic *Escherichia coli* (APEC) from broilerchickens in Sudan.

Taken together this study demonstrated that *E. coli* is a major contaminant in broilers farms and maybe responsible for disease conditions in broiler. Moreover *E. coli* isolates provided resistance to antibiotics used in this study and multi drug resistance (MDR).

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.

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Competing Interest

The authors declared no competing interest.

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