Prevalence of Antibiotic Resistance Genes among *E.coli* Strains Isolated from Poultry in Suez Canal Area

By

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Abstract

A total of 200 cases of diseased and recently dead different poultry species (100 broiler chickens, 50 laying hens, 30ducks and 20 turkeys) with the same prevalence from liver, lung and heart blood were collected from different localities in El- Ismailia Governorate. *E. coli* was isolated from 102(51%) cases. Only representative 10 *E. coli* isolates were serotyped as O111:K58, O1:K1and O146:K99 in order of frequency (60%, 20% and 20%) of the isolates, respectively. Selected representative 13 *E. coli* isolates were tested for their susceptibility to 13 antimicrobial agents and absolute resistance was obtained among selected *E. coli* isolates against amoxicillin clavulanic acid (100%), chloramphenicol (76.9%) and erythromycin (76.9%). In addition, (69.2%) of isolates were resistant to nalidixic acid, rifamycin, streptomycin and cefoxitin and (61.5%) of isolates were resistant to ceftriaxone and all tested isolates were resistant to at least 4 antibiotics and multidrug resistance was seen. The highest sensitivity rates were recorded to ciprofloxacin (84.6%) and colistin sulphate (76.9%). PCR results indicated that representative 10 *E .coli* isolates had antibiotic resistance genes as *dfrA1*, *aada1*, *bla$_{TEM}$* and *Sul1* genes 100 % (10/10), while only 40 % (4/10) had *floR* gene.

Introduction

E.coli as a bacterium is a member of the family *Enterobacteriacae*, facultative anaerobic and gram-negative short rods *(WHO, 1996)*. *E. coli* strains are commensal but some of these bacteria cause intestinal and extra intestinal diseases in humans and animals *(Barnes et al., 2003)*. *E. coli* strains responsible for bird diseases are named avian pathogenic *E.coli* (APEC), and the disease is known as colibacillosis which is a widespread disease that causes great losses in poultry industry *(Barnes et al., 2008)*. The indiscriminate use of antibiotics in the poultry industry as therapeutic agent or feed additive has led to the emergence of multiple drug resistant bacteria *(Mishra et al., 2002)* as there is a high prevalence rate of *E. coli* strains with variable resistance

to a wide range of antimicrobial agents *(Mushi et al., 2008).* Resistance genes transfer horizontally and mediated by plasmids, play a role in the development and dissemination of multidrug resistance *(Yanhong and Wei, 2009).*Recent identification of pathogenic *E.coli* strains needs to detect pathogenic genes in bacterial isolates, allowing the rapid diagnosis of pathogenic *E.coli* as PCR methods using single primer sets have been reported *(Oswald et al, 2000).*

Thus the aim of this study was to investigate antibiotic resistance among *E.coli* strains isolated from poultry

Material and Methods
Collection of samples:

A total number of 200 samples of diseased and freshly dead different poultry species (100 broilers, 50 Laying hens, 30 ducks and 20 turkeys) were collected from different localities at Ismailia province. The diseased birds showed signs of colibacilosis as respiratory distress, reduced feed intake, depressed, growth retardation, decrease in egg production and chick quality and increased mortality with postmortem characteristic lesions (fibrinous exudate covering the heart, fibrinous perihepatitis and septicemia). All samples were collected under aseptic conditions from liver, lungs, and heart blood with the same prevalence.

Isolation of *E. coli*:

It was performed according to *Quinn et al. (1994).* For enrichment one gram of each collected sample was aseptically added to 9ml of buffered peptone water, mixed and incubated at 37ºC for 24hr. A loopful from the incubated broth was streaked on the surface of MacConkey′s agar medium *(Oxoid, CM0007)* plates and incubated at 37ºC for 24hr for primary isolation. Lactose fermenting colonies were picked up and streaked onto EMB agar medium *(Oxoid, CM0069)* plates and incubated at 37ºC for 24hr. Metallic green sheen colored colonies on EMB were subcultured on Nutrient agar slant *(Oxoid, CM0003)* and incubated at 37ºC for 24hr for storage at 4ºC in the refrigerator for further studies and characterization and also in semi-solid agar for preservation as well as for detection of motility.

Identification of isolates:

Suspected *E.coli* isolates were identified morphologically by Gram′s stain and motility test and biochemically by applying the following tests; Oxidase, Methyl Red, Vogues-Proskaur, Indole, Citrate utilization, Nitrate reduction, Urease, TSI and Catalase according to *Qunin et al. (2002), Koneman et al. (1997) and Cruickshank et al. (1975).*

Serotyping:

Selected representative 10 *E. coli* isolates were serotyped by slide agglutination test according to

Edwards and Ewing (1972) at the Reference Laboratory of Veterinary Quality Control on Poultry Production, Dokki, Egypt using commercially available kits with available polyvalent and monovalent anti *E.coli* O and K sera (DENKA SEIKEN, Tokyo, Japan).

Antibiogram:
Antibiotic sensitivity was performed according to *Finegold and Martin, (1982)* using Mueller Hinton Agar plates (**oxoid**) using antibiotic discs of 13 commonly used antibiotics that were obtained from **Kirby-Bauer by (NISSUI), Japan** as recommended by Clinical Laboratory Standard Institute *(CLSI,2015).*

Molecular Identification of *E.coli* isolates:
A total of 10 representive identified *E.coli* strains were tested by specific primer employing PCR assay which was more sensitive in the confirmation of the isolates.

DNA extraction: According to **Emerald Amp GT PCR mastermix (Takara)** Code No. **RR310A** kit.
Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56oC for 10 min.

After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primers:
Primers used were supplied from **Metabion (Germany).**

PCR amplification:
Primers were utilized in a 25- µl reaction containing12.5 µl of **Emerald Amp GT PCR mastermix (Takara, Japan)**, 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of template.The reactions were performed in a thermal cycler-Perkin Elmer/Cetus Research USA

Analysis of the PCR Products:
The products of PCR were separated by electrophoresis on 1.5% agarose gel in 100 ml TBE buffer at room temperature. For gel analysis, 20 µl of the PCR products were loaded to the gel. A 100 bp DNA Ladder **(QIAGEN (USA)** was used to determine the fragment sizes. The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Table (1): Oligonucleotide Primers used for amplification of antibiotic resistant genes of E.coli (agarose gel electrophoresis **(Sambrook et al., 1989).**

Target gene	Primers sequences	Amplified product	Reference
Sul1	F. CGG CGT GGG CTA CCT GAA CG	433 bp	**Ibekwe *et al*., 2011**
	R. GCC GAT CGC GTG AAG TTC CG		
blaTEM	F. ATCAGCAATAAACCAGC	516 bp	**Colom *et al*., 2003**
	R. CCCCGAAGAACGTTTTC		
dfrA	F.TGGTAGCTATATCGAAGAATGGAGT	425 bp	**Grape *et al*., 2007**
	R.TATGTTAGAGGCGAAGTCTTGGGTA		
Aada1	F.TATCAGAGGTAGTTGGCGTCAT	484 bp	**Randall *et al*. 2004**
	R.GTTCCATAGCGTTAAGGTTTCATT		
floR	F.TTTGGWCCGCTMTCRGAC	494 bp	**Doublet *et al*., 2003**
	R.SGAGAARAAGACGAAGAAG		

Cycling conditions of cPCR :

Table (2): *Cycling conditions of the different primers   during cPCR according to Emerald Amp GT PCR Mastermix (Takara) kit.*

Gene	Primary denaturation	Amplification				Final extension
		Secondary denaturation	Annealing	Extension	No. of cycles	
Sul1	94°C 5 min.	94°C 30 sec.	60°C 40 sec.	72°C 40 sec.	35	72°C 10 min.
blaTEM	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 40 sec.	35	72°C 10 min.
dfrA	94°C 5 min.	94°C 30 sec.	60°C 40 sec.	72°C 40 sec.	35	72°C 10 min.
Aada1	94°C 5 min.	94°C 30 sec.	54°C 40 sec.	72°C 40 sec.	35	72°C 10 min.
floR	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 40 sec.	35	72°C 10 min.

Results
Prevalence of *E. coli* isolated from poultry species
One hundred and two *E.coli* strains were recovered from 200 examined samples collected from different poultry from different organs with the same prevalence (51%).

Isolation and Identification of *E. coli* isolates

As regarding to morphological and biochemical characters, isolates appeared as smooth, shiny, strong lactose fermenting colonies on MacConkey's agar and characteristic greenish metallic sheen on EMB agar. All isolates were Oxidase negative, Catalase positive and highly motile. On TSI agar, all isolates produced acid butt

and slant (A/A with CO2 production) without H2S production. The result of IMVC test was (++--) and Urease test negative.

Results of antimicrobial susceptibility testing:

Thirteen representive *E. coli* isolates were selected (the isolates with the code no. (1, 5, 18, 35, 70, 72, 91,102 and117) from broilers, the isolate with the code no. (58) from duck, the isolates with the code no.(2and80) from laying hens and the isolate with the code no.(40) from turkey). All 13 isolates were tested for their susceptibility to 13 antimicrobial agents. The highest sensitivity rate was against ciprofloxacin (84.6%), while absolute resistance was against amoxicillin/ clavulanic acid (100%), as shown in **table (5)**. All 13 isolates were resistant to at least 4 antibiotics and multidrug resistance was seen.

Table (3): *Prevalence of E.coli isolated from examined poultry samples.*

| Type of examined poultry samples	Number of samples	Number of +Ve cases	Prevalence of +Ve cases	Number of -Ve cases	Prevalence of -Ve cases
-Broiler chickens -Laying hens -Ducks -Turkeys -Total	100 50 30 20 200	61 23 11 7 102	61% 46% 36.7% 35% 51%	39 27 19 13 98	39% 54% 63.3% 65% 49%

+Ve= Positive   **-Ve= Negative**

Table (4): *Serotyping of 10 representive E.coli isolates from different poultry species*

Isolate code no.	*E. coli* serotype	Percentage
1 2 40 80 91 117	O111:K58	6/10 (60%)
5 35	O1:K1	2/10 (20%)
18 58	O146:K99	2/10 (20%)

Table (5) *Results of antimicrobial sensitivity testing for representative 13 E. coli isolates*

Antimicrobial discs	No.& % of disc diffusion among 13 *E.coli* isolates					
	Resistant		**Intermediate**		**Sensitive**	
	No.	**%**	**No.**	**%**	**No.**	**%**
Chloramphincol	10	76.9	0	0	3	23.1
Ciprofloxacin	1	7.7	1	7.7	11	84.6
Colistin Sulphate	3	23.1	0	0	10	76.9
Doxycycline	5	38.4	2	15.4	6	46.2
Cefoxitin	9	69.2	1	7.7	3	23.1
Erythromycin	10	76.9	0	0	3	23.1
Gentamycin	5	38.4	0	0	8	61.5
Nalidixic acid	9	69.2	0	0	4	30.8
Rifamycin	9	69.2	1	7.7	3	23.1
Amoxicillin /clavulinicacid	13	100	0	0	0	0
Streptomycin	9	69.2	0	0	4	30.8
Ceftriaxone	8	61.5	2	15.4	3	23.1
Sulfamethoxazole-trimethoprim	8	61.5	3	23.1	2	15.4

Detection of antibiotic resistance genes by PCR in among representative 10 *E.coli* isolates
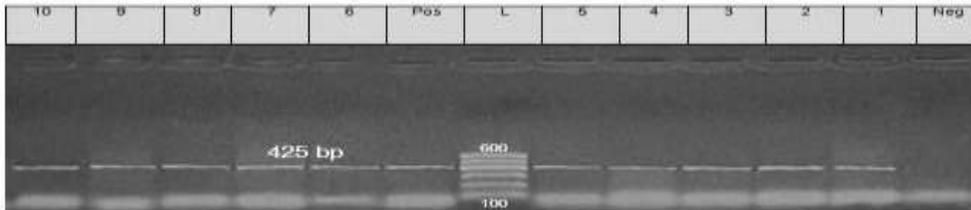

Agarose gel electrophoresis showing the result of PCR for detection of *dfrA* **gene from 10 *E.coli* isolates. Lanes 1,2,3,4,5,6,7,8,9,10:** positive amplification of 425bp for *dfrA* gene of different *E .coli* strains. **L:** Molecular ladder with molecular weight marker (100-600 bp).**Pos:** positive *dfrA* control (reference strain). **Neg :** negative *dfrA* control (control negative).
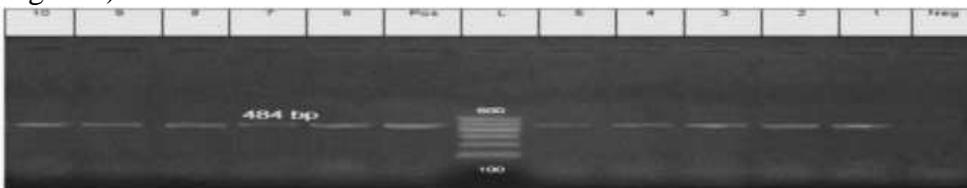
Agarose gel electrophoresis showing the result of PCR for detection of *aada1* **gene from 10 *E.coli* isolates. Lanes 1,2,3,4,5,6,7,8,9,10:** positive

amplification of 484bp for *aada1*gene of different *E. coli* strains. **L:** Molecular ladder with molecular weight marker (100-600 bp). **Pos:** positive *aada1* control (reference strain).**Neg :** Negative control. negative *aada1* control (control negative).

Agarose gel electrophoresis showing the result of PCR for detection of blaTEM gene from 10 E.coli isolates.Lanes 1,2,3,4,5,6,7,8,9,10: positive amplification of 516bp for *bla*$_{TEM}$ gene of different *E.coli* strains.**L:** Molecular ladder withmolecular weight marker (100-600 bp). **Pos:** positive *bla*$_{TEM}$ *control* (reference strain). **Neg:** negative *bla*$_{TEM}$ control (control negative).
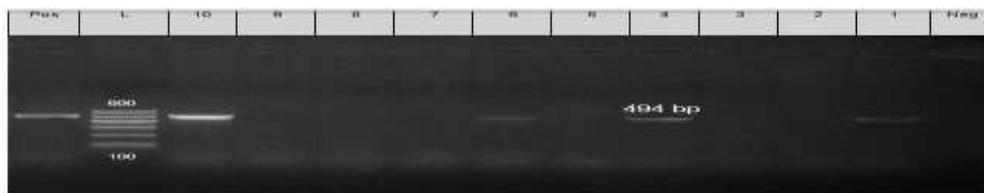
Agarose gel electrophoresis showing the result of PCRfor detection of *floR* **gene from10 *E.coli* isoates.Lanes 1,4,6,10:** positive amplification of 494bp for *floR* gene of different *E.coli* strains. **Lanes 2,3,5,7,8,9:** negative amplification of 494bp for *floR* gene of different *E.coli* strains.L: Molecular ladder with molecular weight marker (100-600 bp). **Pos:** positive *floR* control (reference strain).**Neg:** negative *floR* control(control negative).
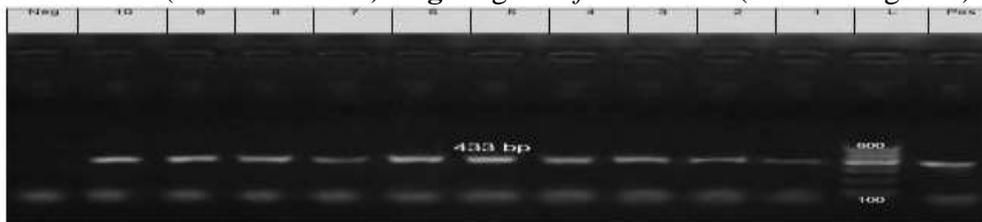
Agarose gel electrophoresis showing the result of PCR for detection of *Sul1* **gene from 10 *E.coli* isolates.Lanes 1,2,3,4,5 ,6,7,8,9,10:** positive amplification of 433bp for *Sul1* gene of different *E.coli* Strains.**L:** Molecular ladder with molecular weight marker (100-600 bp). **Pos:** positive *Sul1* control (reference strain). **Neg :** negative *Sul1* control (control negative).

Table (6) *Association between resistance pattern and genetic profile of E. coli isolates.*

Code no.	Serotype	Antimicrobial resistance pattern			Genomic resistance profile
		R	**I**	**S**	
1	O111:K58	CN,S,AMC,CRO,FOX,C,E,RF	DO, SXT	CIP,CT, NA	*FloR, Sul1, bla$_{TEM}$,aada1,dfrA*
2	O111:K58	CN,S,AMC,NA,CRO,FOX,E,RF,SXT,CT		CIP,C ,DO	*Sul1,bla$_{TEM}$,aada1 , dfrA*
5	O1:K1	CN,S,AMC,NA,CRO,FOX,E,RF,SXT,CT,C,DO	CIP		*Sul1,bla$_{TEM}$,aada1 ,dfrA*
18	O146:K99	S,NA,AMC,CRO,FOX,C,SXT		CN,CIP,DO,E,CT,RF	*FloR,Sul1,bla$_{TEM}$,aada1 ,dfrA*
35	O1:K1	AMC,CRO,FOX,E	DO, SXT	CN,S,CIP,NA,C,CT,RF	*Sul1, bla$_{TEM}$,aada1,dfrA*
40	O111:K58	S,NA,AMC,CRO,FOX,C,RF	SXT	CN,CIP,E,DO,CT	*FloR,Sul1,bla$_{TEM}$,aada1 ,dfrA*
58	O146:K99	S,NA,AMC,C,E,SXT,RF	CRO	CN,CIP,CT DO,FOX	*Sul1, bla$_{TEM}$,aada1,dfrA*
80	O111:K58	CN,S,AMC,NA,CRO,FOX,E,RF,SXT,CIP,C,DO		CT	*Sul1, bla$_{TEM}$,aada1,dfrA*
91	O111:K58	S,AMC,NA,CRO,FOX,E,RF,SXT,C,DO		CN,CIP,CT	*Sul1, bla$_{TEM}$,aada1,dfrA*
117	O111:K58	CN,S,AMC,NA,FOX,E,RF,SXT,CT,C,DO		CRO,CIP	*FloR, Sul1, bla$_{TEM}$,aada1 ,dfrA*

{ C(chloramphenicol), CIP(ciprofloxacin), CT(colistin sulphate), DO(doxycycline), FOX (cefoxitin), E(erythromycin), CN(gentamycin), NA(nalidixic acid), RF(rifamycin) AMC(amoxicillin / clavulinic acid), S(streptomycin), CRO(ceftriaxone) and SXT (trimethoprim /sulphamethoxazole) }.

Discussion

In the present study, *E. coli* was recovered from 102 (51%) out of the total examined 200 diseased and recently dead different poultry species(100 broilers, 50 Laying hens, 30 ducks and 20 turkeys) with the same prevalence from liver, lung and heart blood as shown in **Table (3)**. That agree with *(Abd-El Twab et al., 2015a)* who recovered *E.coli* in (51.1%) of the tested samples. Higher rates were recorded by *(Eid and Erfan, 2013)* who recovered *E.coli* in (80%) of the tested samples. While lower rates were recorded by *(Ammar et*

al., 2015) who isolated *E.coli* in (20%) of the tested samples. Concerning serotyping, *E. coli* represented as 10 strains that were serotyped; 6 as O111:K58 (60%) as the most prevalent serotype among isolates, 2 as O146:K99 (20%) and 2 as O1:K1 (20%). Moreover, the serogroup O146 was positive for K99 (virulence factor). Similar *E.coli* serotypes had been also previously isolated from cases of poultry in Egypt as previously reported *(Shimaa et al., 2013)* concerning to the recently identified serotype O146 in Egypt that agree with *(Eid and Erfan, 2013).*

Concerning antimicrobial susceptibility pattern among representative 13 *E. coli* isolates as shown in **Table (5)**, resistance to amoxycillin/ clavulanic acid was(100%) that agreed with *(Ammar et al.,2015)* who recorded (100%) resistance against amoxycillin/ clavulanic acid. Also, absolute resistance was against both chloramphenicol and erythromycin as (76.9%), also, against rifamycin, cefoxitin and streptomycin was (69.2%) and trimethoprime/sulfamethazone and ceftriaxone was (61.5%). That agree with *(Awad et al., 2016)* who recorded (50%) resistance to streptomycin, (58.6%) to trimethoprime/sulfamethazone and (84.5%) to chloramphenicol.The higher percentages were recorded by *(Ammar et al., 2015)* as (100%) resistance against trimethoprime/sulfamethazone and erythromycin, (98%) for rifamycin and streptomycin, (90%) for chloramphenicol and (84%) for ceftriaxone. In addition ,all tested isolates were resistant to at least 4 antibiotics and multidrug resistance was seen .The study showed high sensitivity rates to ciprofloxacin (84.6%) and to colistin sulphate (76.9%) that agreed with those of *(Eid and Erfan, 2013)* who recorded (75%)and(89.3%) sensitivity rates to ciprofloxacin and to colistin sulphate respectively, but disagreed with those of **(Ammar *et al.,* 2015)** who detected high resistance rates (61%) against

ciprofloxacin and (84%) resistance against colistin sulphate. Also, sensitivity rate to gentamycin and doxycyclin was (61.5%) as shown in **Table (5)** that agree with *(Abd-El Twab et al., 2015b)* who recorded (50%) for gentamycin but not agree with *(Ammar et al., 2015)* who reported (27%) for gentamycin and *(Eid and Erfan, 2013)* who recorded resistance against doxycyclin (100%). Five antibiotic resistance genes (*dfrA* gene, *blaTEM* gene, *aada1* gene, *sul1* gene and *floR* gene) were detected in representative 10 *E.coli* isolates. The data recorded in **Table (6)** revealed that *Sul1* gene showed resistance to sulfamethoxazole , bla_{TEM} gene that correlated with the resistance phenotype to amoxicillin and *aada1* gene that correlated with the resistance phenotype to aminoglycoside (streptomycin) were detected in (100%) of the isolates which indicated the relationship between phenotypic and genotypic features of antibiotic resistance in *E.coli* as shown in **Table (6)** and agreed with *(Ammar et al, 2015)* who found *blaTEM, aad1* and *sul*1 genes in all tested isolates (100%),but not agree with *(Awad et al., 2016)* who found *sul1* gene in only (33.8%)of isolates and *(Shehata et al., 2016)* who found no one of tested *E.coli* isolates contained bla_{TEM} gene. The *dfrA1* gene that encoded resistance to trimethoprim which was detected in 100% of the isolates that not agreed with *(Van et al.,2008)* who found

dfrA1 gene in (26%) of the isolates and *floR* gene which encoded resistance to chloramphenicol was detected in (40%) strains that indicated that this gene was not very well expressed in these isolates as shown in **Table (6)**. This agreed with *(Zhao et al., 2012)* who found *floR* in (43%) of the isolates. These results are signifying that the results of antibiotic disc diffusion test actually agreed with the results of PCR for detection of the relevant antibiotic resistance genes. This study focuses on the correlation between a resistance phenotype and presence of the related genes which was partially displayed in *E.coli* isolates.

Conclusion Based on the present findings, it can be clearly demonstrated that *E.coli* is a major pathogen of poultry in Egypt. There was emerging drug resistance in APEC associated with colibacillosis and the observed high level of multidrug resistance was attributed to a pool of antibiotic- resistance genes and it could hamper the treatment of colibacillosis in Egypt.

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<u>**الملخص العربى**</u>

مدي انتشار الجينات المقاومة للمضادات الحيوية بين عترات الميكروب القولوني المعزول من الطيور في منطقة قناة السويس

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**المعمل المرجعى للرقابة البيطرية على الانتاج الداجنى (الدقى)معهد بحوث صحة الحيوان

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تم جمع عدد200 عينة من الدجاج المصاب والنافق حديثا من اعمار مختلفة من اماكن مختلفة في محافظة االاسماعيلية لفحصها بكتريولوجيا وكيميائيا للكشف عن مدى وجود ميكروب الإيشيريشيا كولاى. حيث تم عزل 102 ميكروب الإيشيريشيا كولاى من 200 عينه بنسبة 51%,كما أظهرت نتائج السيرولوجى لـ10 معزولات أنه قد سادت عترة (O111:K58)بنسبة60% يليها عترة (O1:K1) و(O146:K99)بنسبة20% علي التوالي.تمت دراسة حساسية13 من العترات التي تم الحصول عليها في المختبر للمضادات الحيوية المختلفة بطريقة انتشار القرص.وقد وجد أن غالبية عترات الإيشيريشيا كولاى حساسة للسيبروفلوكساسين بنسبة84,6% و سلفات الكولستين بنسبة76,9% وجميع13 عترة الإيشيريشيا كولاى مقاومه للاموكسيسيلين بنسبة100%.أيضا، كانت جميع العترات مقاومة لاكثرمن4مضادات حيوية والمقاومة للأدوية المتعددة شوهدت في جميع العترات.وجد ارتباط بين النمط الظاهري والنمط الوراثي لعترات الإيشيريشيا كولاى المقاومة للمضادات الحيويه.كما اشارت نتائج تفاعل انزيم البلمره المتسلسل للكشف عن الجينات المقاومه للمضادات الحيويه الى وجود 5 انواع من الجينات المقاومه وهي:(*dfrA* gene, *blaTEM* gene, *aada1* gene, *sul1* gene and *floR* gene).