

Virulence Factors and Genotypic Characterization of *Escherichia coli* Isolated from Chickens

A.A.R.Khafagy*, Samah Eid ** and Rasha A. Mohammed***

Bacteriology, Mycology and Immunology, Department, Faculty of Vet.Med., Suez Canal University*. Bacteriology Department, Animal Health Research Institute, Dokki, Giza**, Egypt 3Reference Laboratory for quality control on poultry production (RLQP). Animal Health Research Institute, Sharkia Branch, Egypt***.

Abstract

This study assesses the presence of one hundred and fourteen *E. coli* strains recovered from five hundred chicken examined samples including five hundred heart, five hundred livers and five hundred cloacal swabs with a percentage of 12.8 %. *E. coli* is one of the most common isolates in avian diseases, which causes colibacillosis, or act as a major factor in development of acute respiratory disease causing high losses especially between chickens. In addition to the conventional methods used for isolation and identification of *E. coli*, PCR is required as rapid, accurate and specific tool for detection of pathogenic *E. coli* and their virulence genes.

Introduction

Escherichia coli is normally found in the digestive tract of poultry and most strains are non-pathogenic. The pathogenic capacity of *E. coli* for chickens to cause significant diarrheal and extraintestinal diseases has been associated with numerous extrinsic and intrinsic bird related factors and condition. The extrinsic factors include environment, exposure to other infectious agents, virulence and duration of exposure. The intrinsic factors affecting susceptibility includes age, route of exposure and breed or strain of chicken *Piercy (1976)*. Avian colibacillosis is regarded as one of the major causes

of morbidity and mortality, associated with heavy economic losses to poultry industry through its association with various disease conditions, either as primary pathogen or as a secondary pathogen *Kwon (2008)*. The symptoms of colibacillosis are non-specific and differ with age, organs involved and concurrent disease. Chickens of all ages are susceptible to colibacillosis but usually young birds are considered more susceptible *Barnes (1997) and Gross (1994)*. It causes a variety of disease syndromes in poultry including yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome,

acute colisepticemia, coligranuloma, enteritis, cellulitis and salpingitis. Colibacillosis of poultry is characterized in its acute form by septicemia resulting death and in its subacute form by pericarditis, airsacculitis and peri hepatitis **Calnek (1997)**. PCR technique is capable of identifying the most highly pathogenic *E. coli* isolates in a flock. **JanBen et al. (2001)**. Based on the fact that virulence varies not only among different species but also among strains of the same species. Thus, numerous studies have been conducted to identify virulence factors of isolated pathogenic *E. coli* strains **Kaipainen et al. (2002); Zaki et al. (2004) and Ewers et al. (2009)**. Avian pathogenic *E. coli* for poultry commonly belong to certain serogroups O1, O2, O11, O15, O55, O78, O79 and O111 **Gross (1994) and Bopp et al. (2005)**. The pathogenic and non-pathogenic strains in poultry are differentiated based on the virulence, which has been attributed to various factors including those encoding for adhesions (F1, P, and stg fimbriae, curli, and EA/I), anti-host defense factors (ompA, iss, lipopolysaccharide, and K1), iron acquisition systems (aerobactin, iroproteins, yersiniabactin, and the sit iron acquisition locus), auto transporters (tsh, vat, and aatA), the phosphate transport system, sugar metabolism, the ibeA protein and motility **Dho-Moulin (1999)**.

This study was planned for bacteriological characterization of chicken *E. coli* isolates and detection of some virulence genes of the isolated strains by using PCR. Therefore, the present study was planned to determine the prevalence and serotypes of avian pathogenic *E. coli* (APEC) strains in broilers farms in winter and summer seasons in Sharkia Governorate, Egypt and detection of some virulence genes of the isolated strains by using polymerase chain reaction (PCR). Thus, the current study was undertaken to:

- 1- Isolate *E. coli* from organs and cloacal swabs samples
- 2- Identify *E. coli* isolates microscopically, biochemically and serologically.
- 3- Detect toxigenic genes (*iss*, *ompA*, *papC*, *tsh*, *iroN*, *eeA*, *sxt1* and *sxt2*) in *E. coli* isolates using PCR.

Material and Methods:

Examination of five hundred samples were collected from different sources in Sharkia province, Egypt. Five hundred samples were collected from (liver, heart and cloacal swabs) of broilers that had died from colibacillosis with typical preceding symptoms like septicemia, respiratory infections and premature death. Samples were aseptically collected in sterile containers and immediately transported in an icebox to the laboratory for further bacteriological examination

according to *Konemann et al. (1997)*, the technique recommended by *Cruickshank et al., (1975)* and *Quinn et al. (2002)* and **MOLECULAR DETECTION OF VIRULENCE GENES**

E. coli isolates were isolated using QIA prep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany). Screening for the presence of virulence genes was carried out by PCR amplifications using specific primers and different cycling conditions as previously described *Sambrook et al. (1989)*. The PCR products were tested for positive amplification by agarose gel electrophoresis. For each PCR experiment, appropriate positive and negative controls were included.

Material used for extraction of DNA

QIAamp DNA Mini Kit Catalogue no.51304

oligonucleotide primers used in cPCR Eight pairs of primers were supplied from metabion (Germany) or Biobasic (Canada).

They have specific sequence and amplify specific products as shown in **Table (1)**.

Material used for Agarose gel electrophoresis (Agarose 1.5%) according to Sambrook et al., (1989)

A multi-purpose, high gel strength agarose suitable for a wide range of molecular biology techniques. As it has high gel strength and exclusion limits, multi AB garose could effectively separate large DNA

fragments with reduced running times. This in turn means less band diffusion, a problem often associated with long running times.

It was prepared as follow:

Agarose 1.5%

Agarose powder (ABgene) 1.5 g

TBE 100 ml

Ethidium bromide solution 10 mg / ml Sambrook et al., (1989)

Ethidium bromide powder (Sigma) 10 mg

Sterile DDW 1.0 ml

It was mixed and stored covered at 4°C It was added to melted agarose to reach a final concentration of 0.1-0.5 µg/ml.

Tris borate EDTA (TBE) electrophoresis buffer (1x) WHO, (2002)

Tris buffer (Fluka) 10.78 g

Boric acid (Fluka) 5.5 g

EDTAdiNA (Winlab) 0.82g

It was brought up to 1 liter with deionized water, pH was checked up. If the pH was out of the range of 8-8.6, a new solution was prepared again.

Any change in ion concentration would affect the migration of the DNA through the gel.

Equipment and apparatuses used in cPCR

Calibrated cylinders, Glass flasks, PCR tubes 0.2 ml capacity, Balance (**Scaltec**), Microwave (**Panasonic**), Monochannel micropipette (2-20 µl) (**Biohit**), Sterile filter tips, Gel casting apparatus (**Biometra**), T3 Thermal cycler (**Biometra**), Power supply (**Biometra**), Type II A biosafety cabinet. (**Thermo**), Gel

documentation system (**Alpha Innotech**), Deionizer (**Millipore**) and Double distillator (**Sanyo**).

Extraction of DNA according to a QIAamp DNA Mini Kit Catalogue no.51304 according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, United States)

Preparation of PCR Master Mix according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit as shown in table (2).

Temperature and time conditions of the primers during PCR are shown in table (3).

DNA Molecular weight marker

The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

Agarose gel electrophoresis (Sambrook et al., 1989) with modification

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE

Table (1): Oligonucleotide primers sequences.

| Gene | Primer sequence (5'-3') | Length of amplified product | Reference |
|-------------|-------------------------------|-----------------------------|---------------------------|
| <i>iss</i> | F:ATGTTATTTTCTGCCGCTCTG | 266 bp | Yaguchi et al., 2007 |
| | R:CTATTGTGAGCAATATACCC | | |
| <i>Tsh</i> | F:GGT GGT GCA CTG GAG TGG | 620 bp | Delicato et al., 2003 |
| | R:AGT CCA GCG TGA TAG TGG | | |
| <i>iroN</i> | F:ATC CTC TGG TCG CTA ACT G | 847 bp | Ewers et al., 2007 |
| | R:CTG CAC TGG AAG AAC TGT TCT | | |
| <i>papC</i> | F:TGATATCACGTCAGTAGC | 501 bp | Wen-jie et al., 2008 |
| | R:CCGGCCATATTCACATAA | | |
| <i>ompA</i> | F:AGCTATCGCGATTGCAGTG | 919 bp | Ewers et al., 2007 |
| | R:GGTGTGGCCAGTAACCGG | | |
| <i>aeA</i> | F:ATG CTT AGT GCT GGT TTA GG | 248 bp | Bisi-Johnson et al., 2011 |
| | R:GCC TTC ATC ATT TCG CTT TC | | |
| <i>Stx1</i> | F:AACTGGATGATCTCAGTGG | 614 bp | Dipineto et al., 2006 |
| | F: CTGAATCCCCCTCCATTATG | | |
| <i>Stx2</i> | F:CCATGACAACGGACAGCAGTT | 779 bp | |
| | R:CCTGTCAACTGAGCAGCACTTTG | | |

DNA Molecular weight marker

buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70°C, then 0.5µg/ml ethidium bromide was added and mixed thoroughly.

The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization.

The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty µl of each uniplex PCR product and 40 µl of each duplex PCR product and negative and positive controls were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet.

The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Gel Pilot 100 bp ladder (cat. no. 239035) supplied from QIAGEN (USA).
Number of bands: 6 Size range: 100-600 bp.

Gene ruler 100 bp DNA ladder (cat. no. SM0243) supplied from Fermentas.

Number of bands: 10 Size range: 100-1000 bp

Table (2): Preparation of PCR Master Mix:

| Component | Volume/reaction |
|--|-----------------|
| Emerald Amp GT PCR mastermix (2x premix) | 12.5 μ l |
| PCR grade water | 4.5 μ l |
| Forward primer (20 pmol) | 1 μ l |
| Reverse primer (20 pmol) | 1 μ l |
| Template DNA | 6 μ l |
| Total | 25 μ l |

Preparation of *stx1*, *stx2* multiplex PCR Master Mix

| Component | Volume/reaction |
|--|-----------------|
| Emerald Amp GT PCR mastermix (2x premix) | 25 μ l |
| PCR grade water | 13 μ l |
| Forward primer (20 pmol) | 1 μ l each |
| Reverse primer (20 pmol) | 1 μ l each |
| Template DNA | 8 μ l |
| Total | 50 μ l |

Table 3: Cycling conditions of the primers during PCR

| Gene | Primary denaturation | Secondary denaturation | Annealing | Extension | No. of cycles | Final extension |
|------------------------------|----------------------|------------------------|-----------------|-----------------|---------------|-----------------|
| <i>iss</i> | 94°C 5 min. | 94°C 30 sec. | 54°C 30 sec. | 72°C 30 sec. | 35 | 72°C 7 min. |
| <i>tsh</i> | 94°C 5 min. | 94°C 30 sec. | 54°C 40 sec. | 72°C 45 sec. | 35 | 72°C 10 min. |
| <i>iroN</i> | 94°C 5 min. | 94°C 30 sec. | 50°C 40 sec. | 72°C 50 sec. | 35 | 72°C 10 min. |
| <i>papC</i> | 95°C 5 min. | 94°C 30 sec. | 58°C 40 sec. | 72°C 40 sec. | 35 | 72°C 10 min. |
| <i>ompA</i> | 95°C 5 min. | 94°C 30 sec. | 58°C 40 sec. | 72°C 1min. | 35 | 72°C 12 min. |
| <i>eaeA</i> | 94°C 5 min. | 94°C 30 sec. | 51°C 30 sec. | 72°C 30 sec. | 35 | 72°C 7 min. |
| <i>stx1</i> , <i>stx2</i> | 94°C 5 min. | 94°C 30 sec. | 58°C 40 sec. | 72°C 45 sec. | 35 | 72°C 10 min. |

Results & Discussion:

Hence, rapid identification of pathogenic *E. coli* strains and detection of their virulence and resistance genes allow the rapid diagnosis of pathogenic *E. coli*, so the use of PCR is a powerful molecular biological technique. It provides rapid, reliable results and shows high sensitivity and specificity in the detection some important virulence genes and resistance genes among *E. coli* isolates (*Eid and Erfan, 2013*).

All isolates were positive for *iss* gene (100%) which had a vital role in *E. coli* pathogenicity and could be a potential target for developing novel therapeutics and prevention strategies. It gave a characteristic band at 266 bp.as shown in **Figure (7)**.

Another gene associated with bacterial virulence is *ompA* gene which is responsible for *E. coli* attachment at the host cell were represented in all *E. coli* isolates (100%) as shown in **Figure (3)**,

papC gene which is involved in adhesion of pathogenic *E. coli* to the host cells produced a pronounced band at 501 bp in all tested *E. coli* isolates except one isolate from fecal swabs as shown in **Figure (5)**. Furthermore; *tsh* gene which had haemagglutinating activity in APEC produced a pronounced band at 620 bp in all tested *E. coli* isolates except two isolate from liver and heart samples as shown in **Figure (6)**,

iroN gene which helps *E. coli* to survive in their host aquatic habitat. Amplification of *iroN* gene produced a pronounced band at 847bp in all tested *E. coli* isolates except three isolate (63 H, 82 H and 75 LV) as shown in **Figure (4)**. additional factors that contribute to virulence including intimin (encoded by the *eae* gene), an outer membrane protein involved in the attachment of *E. coli* to the enterocyte with amplican sizes 248 bp as shown in **Figure (2)**

Besides Shiga toxins *stx1* and *stx2* not found as shown in **Figure (1)**

Our results confirmed the presence of virulence genes including *iss* gene, *ompA*, *papC*, *tsh*, *iroN*, and *eae* gene in chickens **Table (4)**.

Also (*Dutta et al., 2011*) collected 15 strains were analyzed by PCR and detect *stx1*, *stx2* and *eaeA* genes. (*Parreira and Gyles, 2002*) found *stx1* and *stx2* genes in *E. coli* isolated from avian samples. (*Zakeri, 2014*) detected *eaeA* gene in *E. coli* isolated from chicken samples.

Absence of STEC *stx1* and *stx2* gene which get in parallel with *Farooq et al., (2009)* and *Wani et al., (2004)* Similarly, *Schroeder et al., (2003)* could not isolate STEC from retail chicken and turkey obtained from Washington and *Kobayashi et al. (2002)* did not observe STEC in fecal samples from 199 broiler chickens in Finland. The detection of STEC in chicken was in contrast with *EL-*

Jakee et al. (2012) who detected *stx2* by percentage 41.67%.

Concerning to examination of *E. coli* isolates for the presence of Intimin gene, results detected 2 out of 12 *E. coli*. These findings were nearly agreed with those obtained from (**Dutta et al., 2011**) who detected *eaeA* in 4 *E. coli* strains out of 10 isolates and (**El- Jakee et al., 2012**) who detected *eaeA* in 5 *E. coli* strains out of 12.

***stx1* and *stx2* genes**

lanes (1- 12) represent the tested strains, PCR failed to detect *Stx1* and *stx2* genes in tested isolates. positive control (Lane. pos) and negative control (Lane. neg).

positive controls represented by field sample that were previously confirmed to be positive by PCR for *Stx1* and *Stx2* genes in Reference laboratory for veterinary quality control on poultry production, Animal health research institute.

negative controls represented by buffer without DNA.

***eaeA* gene**

lanes (1- 12) represent the tested strains, Lanes 2,7 *E. coli* isolate (code No. 22H and 73 LV) showed *eaeA* gene positive PCR products. positive control (Lane. pos) and negative control (Lane. neg).

positive controls represented by field sample that were previously confirmed to be positive by PCR for *Stx1* and *Stx2* genes in Reference laboratory for veterinary quality control on poultry

production, Animal health research institute.

negative controls represented by buffer without DNA

***ompA* gene**

lanes (1- 12) represent the tested strains, Lanes

1,2,3,4,5,6,7,8,9,10,11,12 *E. coli* isolates (code No. 1H, 22 H, 63 H, 82 H, 42 LV, 72 LV, 73 LV, 75 LV, 52 F, 76 F, 8F, 95 F) showed *ompA* gene positive PCR product

positive control (Lane. pos) and negative control (Lane. neg).

***IroN* gene**

lanes (1- 12) represent the tested strains, Lanes 1,2, 5,6,7, 9,10,11,12 *E. coli* isolates (code

No. 1H, 22 H, 42 LV, 72 LV, 73 LV, 52 F, 76 F, 8F, 95 F) showed *IroN* gene positive PCR product. while line number (3,4, 8) (code No. 63 H, 82H, 75 LV) were negative.

positive control (Lane. pos) and negative control (Lane. neg)

***papC* gene**

lanes (1- 12) represent the tested strains, Lanes 1,2,3,4,5,6,7,8, 10,11,12 *E. coli* isolates (code No. 1H, 22 H, 63 H, 82 H, 42 LV, 72 LV, 73 LV, 75 LV, 76 F, 8F, 95 F) showed *PapC* gene positive PCR product. while lane number (9) (code No. 52F) was negative. positive control (Lane. pos) and negative control (Lane. neg)

***tsh* gene**

lanes (1- 12) represent the tested strains, Lanes 1,2,3, 5,6,7, 9,10,11,12 *E. coli* isolates (code No. 1H, 22 H, 63 H, 42 LV,72 LV, 73

LV, 52 F, 76 F, 8F, 95 F) showed *Tsh* gene positive PCR product positive.while lane number (4,8) (code 82H and 75LV) were negative.

Positive control (Lane. pos) and negative control (Lane. neg)

***iss* gene**

lanes (1-12) represent the tested strains,

Lanes 1,2,3,4,5,6,7,8,9,10,11,12 *E. coli* isolates (code No. 1H, 22 H, 63 H, 82 H, 42 LV, 72 LV, 73 LV, 75 LV, 52 F, 76 F, 8F, 95 F) showed *iss* gene positive PCR product. positive control (Lane. pos) and negative control (Lane. neg)

E. coli is one of the most common isolates in avian diseases, which causes colibacillosis, or act as a major factor in development of acute respiratory disease causing high losses especially between chickens. In addition to the conventional methods used for isolation and identification of *E. coli*, PCR is required as rapid, accurate and specific tool for

detection of pathogenic *E. coli* and their virulence genes.

From the current study, the following points could be concluded:

□□ Bacterial examination confirmed cases of colibacillosis from which 114 *E. coli* strains were isolated.

□□ All isolates had characteristic biochemical features of *E. coli*.

□□ PCR technique leads to an early diagnosis on the pathogenicity of APEC strains, reducing the time of 3-7 days to achieve by conventional techniques to maximum 24 hours.

□□□□□□□□ This study confirmed that *E. coli* is known as one of the most important pathogenic agents causing disease in poultr. The pathogenicity of the strain is caused by presence of at least six virulence genes as *ompA* (outer membrane protein) gene, *iss* gene, *papC* gene, *tsh* gene, *iroN* gene and *eaeA* (intimin or *E. coli* attaching and effacing) gene

***stx1* and *stx2* genes**

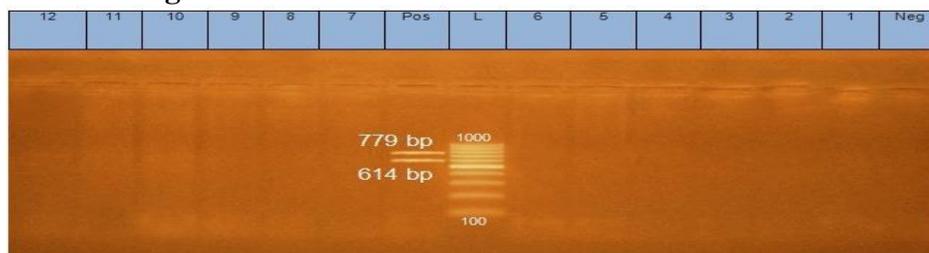


Figure (1): Electrophoretic pattern of the PCR products of *stx1* and *stx2* genes of *E. coli*

Stx1 at (614 bp) and *stx2* at (779 bp) genes Lane L: Ladder 100 bp (100-1000),

***eaeA* gene**

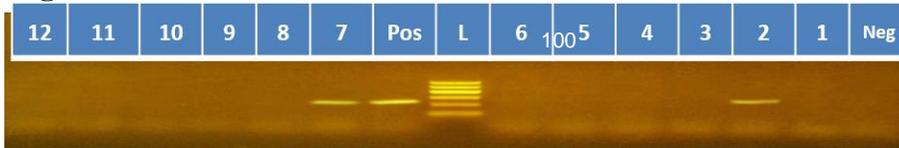


Figure (2): Electrophoretic pattern of the PCR products of *eaeA* gene of *E. coli*. *eaeA* gene at (248bp). Lane L: Ladder 100 bp (100-600),

***ompA* gene**

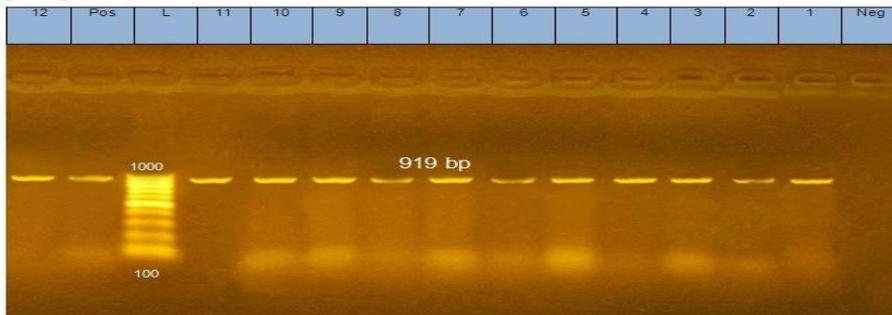


Figure (3): Electrophoretic pattern of the PCR products of *ompA* gene of *E. coli*. *ompA* gene at (919bp). Lane L: Ladder 100 bp (100-1000),

***IroN* gene**

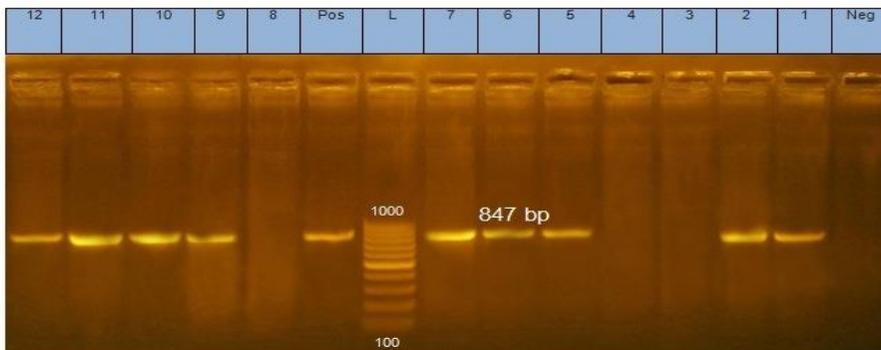


Figure (4): Electrophoretic pattern of the PCR products of *iroN* gene of *E. coli*. *iroN* gene at (847bp). Lane L: Ladder 100 bp (100-1000),

***papC* gene**

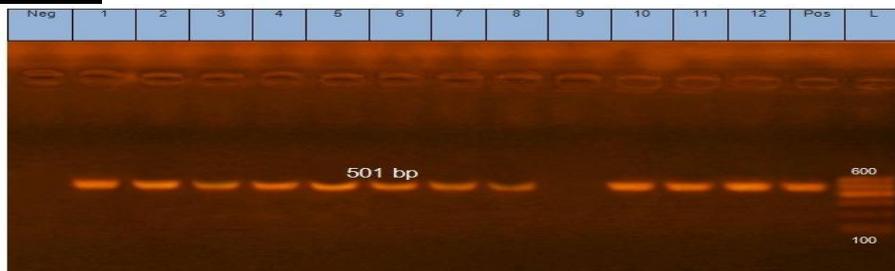


Figure (5): Electrophoretic pattern of the PCR products of *papC* gene
PapC gene at (501bp). L: Ladder 100 bp (100-600),

***tsh* gene**

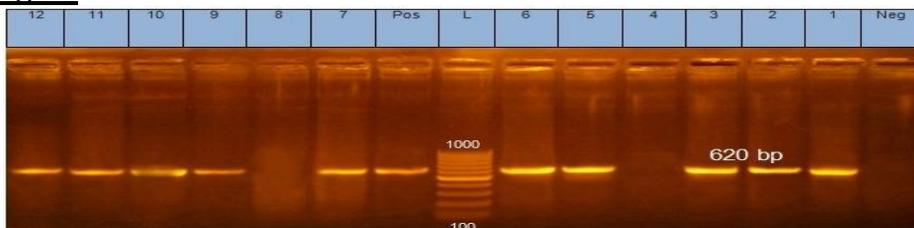


Figure (6): Electrophoretic pattern of the PCR products of *tsh* gene of *E. coli*
tsh gene at (620bp). Lane L: Ladder 100 bp (100-1000),

***iss* gene**

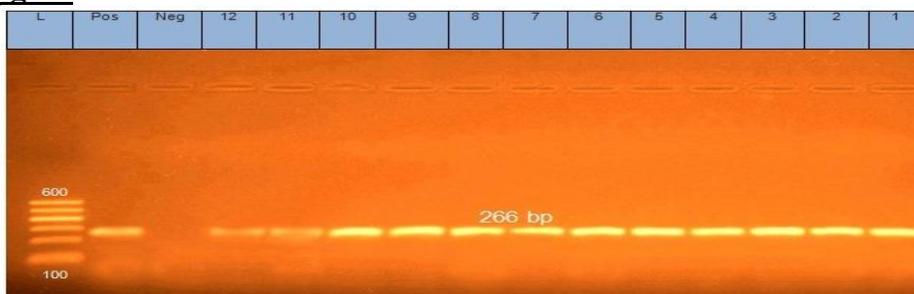


Figure (7): Electrophoretic pattern of the PCR products of *iss* gene of *E. coli*
iss gene at (266bp). Lane L: Ladder 100 bp (100-600),

Table (4): Distribution of virulence genes detected in *E. coli* strains isolated
from both liver & heart blood and fecal swabs samples

| Sample | ID | Serotype | Type | <i>stx1</i> | <i>stx2</i> | <i>eaeA</i> | <i>ompA</i> | <i>iroN</i> | <i>papC</i> | <i>tsh</i> | <i>iss</i> |
|--------|----|-----------|------|-------------|-------------|-------------|-------------|-------------|-------------|------------|------------|
| 1 H | 1 | O44 : H11 | EPEC | - | - | - | + | + | + | + | + |
| 22 H | 2 | O78 : H4 | EPEC | - | - | + | + | + | + | + | + |
| 63 H | 3 | O91 : H11 | EHEC | - | - | - | + | - | + | + | + |
| 82 H | 4 | O1 : H11 | EPEC | - | - | - | + | - | + | - | + |
| 42 LV | 5 | O128: H6 | ETEC | - | - | - | + | + | + | + | + |
| 72 LV | 6 | O91 : H11 | EHEC | - | - | - | + | + | + | + | + |
| 73 LV | 7 | O2 : H2 | EPEC | - | - | + | + | + | + | + | + |
| 75LV | 8 | O125 | ETEC | - | - | - | + | - | + | - | + |
| 52F | 9 | O2 : H7 | EPEC | - | - | - | + | + | - | + | + |
| 76 F | 10 | O78 : H4 | EPEC | - | - | - | + | + | + | + | + |
| 8 F | 11 | O121 : H6 | EHEC | - | - | - | + | + | + | + | + |
| 95 F | 12 | O78 : H4 | EPEC | - | - | - | + | + | + | + | + |

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الملخص العربي

"عوامل الضراوة والتصنيف الجيني لميكروب الايشيريشيا كولاى المعزوله من الدجاج " تعد الإيشيريشيا كولاى احدى الكائنات الدقيقة الطبيعية الموجودة فى أمعاء الدواجن ولكن بعض السلالات تمتلك عوامل الضراوة وتسبب مرض الكولي باسيلوزيس والذي يعد أحد أهم الأمراض التي تصيب الدواجن و يؤدي الي خسائر اقتصادية فادحة في صناعة الدواجن في أنحاء كثيرة من العالم، لذا تهدف هذه الدراسة لتحديد مدى انتشار الإيشيريشيا كولاى و مناقشة توزيع جينات الضراوة عبر الإيشيريشيا كولاى المعوية المنزفة في الدجاج .

تم تجميع خمسمائه عينة من الدجاج (ا المريضة) والتي تعاني من التهاب غشاء التامور ، التهاب حوائط الكبد ، و التهاب الاكياس الهوائية في محافظة الشرقية . وقد خضعت كل العينات للعزل و التصنيف البيوكيميائى للايشيريشيا كولاى. وقد تبين بالتحليل البكتيرى تواجد الميكروب القولونى بنسبه (٦٤ ٪) من العينات التي تم جمعها. تم إجراء تفاعل إنزيم البلمرة المتسلسل على خمسة عشر عترة من الإيشيريشيا كولاى للكشف عن ثمانية جينات ضراوة والتي لها دورهام فى ضراوة عترات الإيشيريشيا كولاى وكانت هذه الجينات *stx1* و *stx2* و *eaeA* و *papC* و *iroN* و *tsh* و *ompA* و *iss* . وقد أعطى كل جين منطقة مميزة عند الوزن الجزيئى

eaeA gene 248 bp , *ompA* gene 919 bp, *iroN* gene 847 bp, *papC* gene 501 bp, *tsh* gene 620 bp and *iss* gene 266 bp

بينما كلا من *stx1* و *stx2* سلبي في كل العينات. وقد اوضحت الدراسات ان نسبة الكشف عن كلا من جين *iss* و *ompA* 100% بينما *papC* و *tsh* gene يليهم *iroN* ثم *eaeA* كانت نسبتهم علي التوالي ٩١,٦ ٪ و ٨٣,٣ ٪ و ٧٥ ٪ و ١٦,٧ ٪ .