# Virulence and Resistance genes of Gram Negative Bacteria Escherichia coli and Pseudomonas aeruginosa in Poultry Products and Edible Egg

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**Abstract:** This work was performed for determination of virulence and resistance genes of *E coli* and *Pseudomonas aureginosa* isolated from poultry products and edible eggs. A total of 150 poultry products represented as chicken fillet, chicken liver, smoked chicken products (50 for each) and 90 red egg samples were analyzed bacteriologically by classical cultural methods. All isolated bacteria were identified using standard microbiological identification technique. The antimicrobial susceptibility profile of the isolates was determined by Kirby –Baur disk diffusion technique. Virulence and resistance genes were determined by using PCR technique. Overall samples, E coli was detected in poultry products in chicken fillet (14) 28% chicken liver (5) 10% smoked chicken (0) 0% and red egg (8) 26.6%. *Pseudomonas spp* was detected as follow from poultry fillet (6) 12% and from egg samples (4) 13.3%. Shiga toxins (*stx*1, *stx*2) were not detected in this study but *eae* A gene was detected in all isolated *E coli* strains while $bla_{Tem}$ and *tet*A were detected in only one strain of *E coli* while *erm*B gene failed to be detected. For pseudomonas confirmed by using 16srRNA gene and detection of virulence genes as *las*B, *tox*A and resistance genes as *mex*R genes were detected in all tested isolates while *bla* vim not detected in the examined isolates.
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**Key words:** *E. coli, P aeruginosa*, Poultry products, Virulence, Resistance genes.

## 1. Introduction

*E.coli* and Pseudomonas aeruginosa are considered common bacterial pathogens that are implicated in most food borne infection **(Iroha *et al.*, 2016)**. Consumption of poultry products and edible egg also improper handling of egg and egg products considered as a source of food borne diseases, such as *E coli*, *Salmonella* and campylobacter **(Heriskstad *et al.*, 2002)**. Though *E coli* lives inside gastrointestinal tract of bird but under certain factors as inadequate ventilation, overcrowding, hunger and extreme temperature it converts to pathogenic and may contaminate carcass during slaughter **(Kaul *et al.*, 1992)**.

Direct contact in human population with poultry has been linked to be a severe source of infection with drug resistant organisms. **(Molbak *et al.*, 1999)**. Pseudomonas *aeruginosa* is a ubiquitously distributed opportunistic Gram-negative pathogen that inhabits soil and water, colonizing plant, animal and human hosts. In food microbiology it is one of the major spoilers, being a marker of hygiene qualities of food and water for human consumption **(Neves, 2014 ) and Pier and Ramphal (2005)**.

Pseudomonas *aeruginosa* is a classic opportunistic pathogen because of its innate resistance to many antibiotics and disinfectants. Also It is the most common Gram negative bacterium found in nosocomial infections **(Devan and Saxena 2013)**. It is commonly discovered in water, soil, and plants. It rarely causes illness in healthy people.

Detection of virulence genes and resistance genes using PCR is highly specific and more sensitive method and more important for requiring a less time procedure **(Malkawi, 2003)**.

## 2. Material and Methods

### 2.1. Samples collection

A total of 150 poultry products samples including chicken fillet (n=50), chicken liver (n=50), smoked chicken products (n=50) edible red eggs (n=30) "represented as 30 egg content and 30 egg shell". These samples were being transferred without delay to the laboratory in an ice box under sanitary condition to the laboratory for bacteriological examination within limited time.

### 2.2. Bacteriological analysis

One gram of each sample was inoculated into nutrient broth (Oxoid UK) tubes and then incubated at 37$^{o}$C for 24h After that tubes that show turbidity were cultured on MacConkey agar (MAC) for the isolation and differentiation of lactose and non lactose fermenter bacteria. The culture plates were incubated at 37$^{o}$C for 24h. After that one loopful cultivated on

Eosin Methelene blue (EMB), another one on Pseudomonas cetrimide agar (PCA). All culture media were procured from (Oxoid UK). the suspected colonies were further sub cultured on to freshly prepared media, And the isolated organisms were purified on nutrient agar plates for further bacteriological tests.

**2.3. Preparation of egg samples egg samples:**

Total of 90 samples represented as each 3 egg represent one sample (n=30) which is rinsed by peptone water 1%, then incubated for 24 h and then cultivated on corresponding media after that the egg was sterilized by alcohol and flamed carefully and then broken by sterilized forceps into clean sterilized plastic bags for further bacteriological examination of egg content.

**2.4. Identification of Bacteria**

Suspected colonies were identified using standard microbiological identification techniques including motility test, indole, trible sugar iron test, $H_2S$ production test, sugar fermentation test, citrate utilization test, voges–proskauer test, oxidation-reduction test and Methyl-red test. **(Cheesbrough, 2000).**

**2.5. Antibiogram**

Antimicrobial susceptibility studies were carried out according to the guide of Clinical Laboratory Standard Institute (CLSI, 2016) using the Kirby-Bauer disk diffusion method. Single antibiotic discs including antibiogram assay of isolates: colistin (CT) (10mg), imipenem (IPM) (10mg), amoxycillin clavulenic acid (AMC) (30mg), gentamycin (CN) (10mg), ciprofloxacin (CIP) (5mg), doxycyclin (DO) (5mg), erythromycin (E) (15mg) and cefotaxime (CTX) (30mg). All the antibiotic discs were procured from (Oxoid UK). The test isolate adjusted to 0.5 McFarland turbidity standard were aseptically swabbed into the surface of Muller-Hinton (MH) agar plates, and the antibiotic discs were aseptically inserted into MH agar using sterile forceps. And then the plates were incubated at $37^0$C for 24h.

**2.6. Serology**

By using Diagnostic polyvalent, monovalent antiserum. (Denka Seiken Co., LTD) Japan & (Pro-lab diagnostic, U.K).

2.7. **Detection of Virulence and resistance genes were determined by PCR**:

DNA extraction: by using The QIAamp DNA Mini Kit, PCR master mix and cycling condition of primers during PCR was prepared according to Emerald AMP. GTPCR master mix (TAKARA), oligoneoclotides primers used in PCR have a specific sequence and amplify a specific product as shown Table (1).

DNA samples were amplified in a total of 25 ml as follow 12.5 µl of Emerald AMP GTPCR master mix,1ml of each primer of 20 pmol. concentration of 4.5 ml of water and 6 ml of tempelate, the reaction was performed in isometra thermal cycler, Temprature and time condition of the primer during PCR were applied in table ( 2 ).

Aliquots of amplified PCR product were electrophoresed in 1.5% agarose gel (ABgene) in TBE buffer at room tempreture for gel analysis.

A100 pb ladder of DNA (QIAGenI, VAlechia, cA, USA ) wasused to determine the fragment size and the gel electrophoresed by gel documentation system and the data photographed and analysed by computer soft ware.

**Table (1): Oligonucleotide primers sequencesfor used genes.**

| Target | Gene | Primer sequence (5'-3') | Length of amplified product | Reference |
|---|---|---|---|---|
| *E. coli* | *blaTEM* | ATCAGCAATAAACCAGC<br>CCCCGAAGAACGTTTTC | 516bp | **Colom *et al*., 2003** |
| | *Stx1* | ACACTGGATGATCTCAGTGG<br>CTGAATCCCCCTCCATTATG | 614 bp | **Dipineto*et al.*, 2006** |
| | *Stx2* | CCATGACAACGGACAGCAGTT<br>CCTGTCAACTGAGCAGCACTTTG | 779 bp | |
| | *eaeA* | ATGCTTAGTGCTGGTTTAGG<br>GCCTTCATCATTTCGCTTTC | 248 bp | **Bisi-Johnson *et al*., 2011** |
| | *TetA (A)* | GGTTCACTCGAACGACGTCA<br>CTGTCCGACAAGTTGCATGA | 576 bp | **Randall *et al.* 2004** |
| | *ermB* | CATTTAACGACGAAACTGGC<br>GGAACATCTGTGGTATGGCG | 425 bp | **Schlegelova*et al*., 2008** |
| | *16S rRNA* | GGGGGATCTTCGGACCTCA<br>TCCTTAGAGTGCCCACCCG | 956 bp | **Spilker*et al*., 2004** |
| *P. aeruginosa* | *mexR* | GCGCCATGGCCCATATTCAG<br>GGCATTCGCCAGTAAGCGG | 637 bp | **Sánchez*et al*., 2002** |
| | *lasB* | ACAGGTAGAACGCACGGTTG<br>GATCGACGTGTCCAAACTCC | 1220 bp | **Finnan*et al*., 2004** |
| | *toxA* | GACAACGCCCTCAGCATCACCAGC<br>CGCTGGCCCATTCGCTCCAGCGCT | 396 bp | **Matar*et al*., 2002** |
| | *blaVIM* | TTTGGTCGCATATCGCAACG<br>CCATTCAGCCAGATCGGCAT | 500 bp | **Amudhan*et al.*, 2012** |

**Table (2): Temperature and time conditions of the primers during PCR:**

| Target MO | Gene | Primary denaturation | Secondary denaturation | Annealing | Extension | No. of cycles | Final extension |
|---|---|---|---|---|---|---|---|
| *E. coli* | *blaTEM* | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| | *stx1, stx2* | 94˚C 5 min. | 94˚C 30 sec. | 58˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| | *eaeA* | 94˚C 5 min. | 94˚C 30 sec. | 51˚C 30 sec. | 72˚C 30 sec. | 35 | 72˚C 7 min. |
| | *tetA (A)* | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| | *ermB* | 94˚C 5 min. | 94˚C 30 sec. | 51˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| | *16SrRNA* | 94˚C 5 min. | 94˚C 30 sec. | 52˚C 40 sec. | 72˚C 50 sec. | 35 | 72˚C 10 min. |
| | *mexR* | 94˚C 5 min. | 94˚C 30 sec. | 57˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| *P. aeruginosa* | *lasB* | 94˚C 5 min. | 94˚C 30 sec. | 54˚C 40 sec. | 72˚C 1 min. | 35 | 72˚C 12 min. |
| | *toxA* | 94˚C 5 min. | 94˚C 30 sec. | 55˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |
| | *blaVIM* | 94˚C 5 min. | 94˚C 30 sec. | 50˚C 40 sec. | 72˚C 45 sec. | 35 | 72˚C 10 min. |

## 3. Results

### 3.1. Incidence of *E. coli* in tested samples:

From chicken fillet,14 samples show positive results for harboring *E coli,*5 from chicken liver are positive for *E coli* but smoked chicken products show negative results for containing *E coli*.

### 3.2. *Results of E.coli serogrouping*

The isolated serotypes were as follows from fillet 6 strains were O148, 4 were O125 and 4 strains were O26 respectively. The serotypes from liver samples were 3 strains O125 and 2 strains were O158. From egg samples *E coli* were detected in 8 samples which were serotyped as O1, O55, O44 and O125 respectively.

### 3.3. Antibiotic sensitivity of isolated e coli:

E coli was sensitive for ciprofloxacin, colistin, amoxicillin clavulinic acid and gentamycin but resistant to doxycycline and cefotaxieme, also complete resistance to erythromycin for all tested isolates. One strain from tested *E coli* show MDR to more than 3 antibiotic.

### 3.4. Virulence and resistance genes of E coli:

Shiga toxins 1,2 weren't detected in isolated strains, eaeA gene was founded in all tested samples as shown in table (3).

Resistance genes as tetA and bla tem were detected in the tested sample while ermB gene was not detected.

**Table (3): Virulence and resistance genes of isolated strains of e coli samples:**

| E. coli sample | Results *bla*TEM | *tet*A | *erm*B | *Stx*1 | *Stx*2 | *eae*A |
|---|---|---|---|---|---|---|
| 1 | Nd | Nd | Nd | - | - | + |
| 2 | Nd | Nd | Nd | - | - | + |
| 3 | Nd | Nd | Nd | - | - | + |
| 4 | Nd | Nd | Nd | - | - | + |
| 5 | Nd | Nd | Nd | - | - | + |
| 6 | + | + | - | - | - | + |

Nd: Not done

### 3.5. Incidence of Pseudomonas from tested samples:

From 50 chicken fillet 6 positive samples were obtained with a percent 12% and the isolated strains were P.aureginosa.

Pseudomonas aureginosa were founded in from 4 egg samples with a percent 13.3%. Egg content show negative for isolation of any of examined microorganisms.

### 3.6. Antibiotic sensitivity testing result:

Pseudomonas show sensitivity to ipimenem, gentamycin. ciprofloxacin and amoxycillin clavulinic acid, colistin sulphate, but the isolates were resistant to cefotaxieme, for doxycycline show great degree of resistance.

**3.7. Results of virulence and resistance genes in pseudomonas aeruginosa:**

Virulence genes of pseudomonas 16srRNA, las B and toxA were detected in all samples, also resistance genes mexR was detected in all tested pseudomonas samples while bla vim not detected in all tested samples. As in table (4).

**Table (4): virulence and resistance genes of tested Pseudomonas aeruginosa samples.**

| P. aeruginosa sample | Results 16S rRNA | mexR | blaVIM | lasB | toxA |
|---|---|---|---|---|---|
| A | + | + | - | + | + |
| B | + | + | - | + | + |
| C | + | + | - | + | + |
| D | + | + | - | + | + |

**4. Discussion**

Escherichia coli strains are normal inhabitant in the gut of food animals, which can contaminate the carcass and enter the food supply chain during slaughtering. Generally the presence of *E. coli* in the examined chicken meat and chicken meat products is considered as an indicator for improper handling or unhygienic conditions, also the fact that chicken fillet, thigh and chicken liver samples were not exposed to heat treatment or freezing and considered as raw products, as well as manual evisceration and unsatisfactory hygienic measures of handling and processing are the main reasons for contamination of chicken carcasses **Hashim (2003).**

In this study *E coli* was detected in 14(28%) samples out of 50 fillet samples, 5(10%) from 50 chicken liver and 0 (0%) from 50 smoked chicken samples, nearly similar to **Chaiba *et al.*, (2007)** and ***Ouf-Jehan (2001)***, While **Lee *et al*., (2009)** and **Amira *et al*., (2017**) found *E coli* percent was 4% and 16.7%respectively.

Eggs are valuable as a source of protein. In fact, egg protein is used as standard against which the quality of other food proteins is measured **(Drewnowski, 2010)**. Egg Contamination by microorganisms means breaking of egg quality which may lead to spoilage consequently economic losses and may be transmission of pathogens that induce cases of food borne infection or intoxication to consumers constituting public health hazard **(California egg commission, 1999).**

In this study e coli was detected in red table egg in (8/30) with 26.6% and the highest values of the microbial load of the eggshell may be attributed to contact with exterior environment, the soil, birds that are allowed to range outdoors are more likely to be insects and rodent infestation, bad storage conditions in store house, wrong show in market, high temperature, dust, hand touching, and all other surrounding pollution**. Abdullah (2010) and Galis *et al*., ( 2012)**.

In chicken fillet and liver the isolated strains were O148, O125, O26, O158, O1, O55, O44 respectively and O1, O55, O44 and O125 respectively in egg samples. This results nearly similar to the investigation by **Amira *et al.*, (2017)**.

In the current study *E coli* show 100% sensetivity for ciprofloxacin, amoxycillin clavulinic acid and gentamycin respectively. But the isolates show 30%,60% sensetivity for doxycycline and cefotaxieme, but 90% and 100%resistance for colistin sulphate and erythromycin respectively. This agreed with obtained by **White *et al*., (2002).** Only one strain show resistance for more than one antibiotic discs.

In the current study *eae*A gene which help in adhesion and colonization of *E coli* to hosts was detected in all *E coli* isolates, but *stx*1and *stx*2 not detected in this investigation, this is dissimilar to **Mohammed *et al*., (2016)**, but **Maha (2013)** show presence of *eae* A and stx2 genes, but *stx*1 gene**s** not detected. As mentioned in table (3).

In this study *tet*A and *bla*Tem genes detected and this agree with **Martínez-Vázquez *et al.*, (2018)** and **Li *et al.*, (2017)** That explains the resistance of to ampicillin, tetracycline.

There for their must occur great regulation in use of veterinary antibiotic drug in order to decelerate the advance of antimicrobial resistance **Alonso *et al*., (2017)**. Resistance to ampicillin, streptomycin, tetracycline and sulphatrimethoprim were associated to efflux pump by detection of *bla* tem and *tet*A were the most detected genes **Grand *et al* (2016).**

*Pseudomonas* species are a significant food spoilage problem in refrigerated meat, fish, shell fish and dairy products. Because *Pseudomonas* thrives in water systems, they can be the source of contamination in the food and beverage industry **Siegrist (2007).**

Presence of Pseudomonas in products is minimum at the first of contamination but it become the dominant at the end of shelf life of products and it decrease its shelf life, it considered indicator of food quality also it is one of food poisoning microorganisms **Abdel Aziz (2016).**

For pseudomonas isolation in the current study it was detected in chicken fillet in 6 (n=50) samples by a percent of 12% and the isolated strain was P.aureginosa while **Dilek and Samver (2007)** found only two samples of examined chicken samples were positive for pseudomonas aureginosa, but **Hinton *et al.*, (2007)** and **Iroha *et al*., (2011)** failed to detect pseudomonas from all chicken samples. Red eggs samples 4 positive samples from (n=30) samples were detected by apercent of 13.3% also the isolated strain was *P.aureginosum* while **Maha *et al*., (2013)** could detect pseudomonas from table egg with percentage of 28%, from red eggs.

*Pseudomonas aeruginosa* is a classic opportunistic pathogen because of its innate resistance to many disinfectants and antibiotics. It is also the most common Gram negative bacterium found in nosocomial infections **Devan and and Saxena (2013)**. Determination of pseudomonas by using PCR is performed by using 16srRNA gene which is confirmed as done by **spilker et al., (2004)**

All *P. aeruginosa* isolates were sensitive to imipenem, which is agreed with other studies carried in Egypt as lowest resistance rates was recorded for imipenem (0%) by **Hassuna *et al*., (2015)**. On the other hand, other study carried by **Zafer *et al*., (2014)** in Cairo and found that a high frequency of imipenem resistance and 100% sensitivity for ciprofloxacin.

*P.aeruginosa* also has a large number of virulence factors such as exotoxin A, exoenzyme S, nan 1 and Las genes and in the current study detection of *las* B and *exo*A genes was performed as in table (4). Significant correlations between some virulence genes and source of infections indicates implementation of infection control measures that will help in controlling the dissemination of virulence genes among P. aeruginosa isolates **Khattab *et al*., (2015)** Pathogenicity of *P. aeruginosa* is clearly multifactorial. LasB is one of the most important proteases of *P. aeruginosa*, and in this study detection of these virulence gene *las* Band *tox*A were detected with percent of 100% of all isolates,, this is nearly similar to other studies done by **Habibi and Honarmand (2015)** and **Dadmanesh, *et al.*, (2014)**.

Resistance in Pseudomonas is resulting from change in gene expression as exemplified by mex drug efflux pump and in the current study mexR was represented as 100% in all isolates which play agreat role in resistance to ciprofloxacin, ampicillin and other antibiotics. While *bla* vim failed to be detected in this research. this agree with **Dumas et al., (2006)**.

**Conclusion**

It could be concluded that consumers should take care during handling of poultry products and edible eggs as it harbor very dangerous bacteria that may affects his health, also antibiotics should be used in a proper manner to avoid resistant bacteria that not affected by in treatment.

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