Phenotypic and genotypic studies on *Escherichia coli* strains isolated from food products of animal origin

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Abstract

Foodstuffs of animal origin may present hazards, due to bacterial contamination. This study was conducted to determine *Escherichia coli* in some types of animal source foods (raw milk, raw beef meat and raw poultry meat). Bacteriological examination of total 246 raw food samples of animal origin, raw milk, (105); beef meat (31) and poultry meat (110) were collected from different localities in Ismailia City showed that 64/246 (26.01%) of samples were infected with *E. coli*. Serological identification of (10) *E. coli* strains revealed that they were belonged to *O*\(_{111}\) polyvalent 1; *O*\(_{44}\) polyvalent 2 (2 strains for each); *O*\(_{78}\) polyvalent 4; *O*\(_{146}\) polyvalent 2; *O*\(_{26}\) polyvalent 1; *O*\(_{119}\) polyvalent 1; *O*\(_{86}\) polyvalent 1 and *O*\(_{18}\) polyvalent 3 (1 strain for each). *E. coli* strains showed high susceptible rate to Ciprofloxacin (CIP), (100%) and high resistance (100%) to Ampicillin (AMP), Metronidazole (MTZ), Amoxicillin (AML), and Vancomycin (VAS). Four strains of the isolated *E. coli* were submitted to molecular studies for detection of the *eaeA* (protein intimin), *iutA* (encoding aerobactin) and *iss* (increased serum survival protein) genes, by using PCR technique.

Introduction

Foods and microorganisms have long and interesting association which developed longer before the beginning of recorded history (*Dilbaghi and Sharma, 2007*). Since its discovery in 1805, *E. coli* was considered a harmless, Gram-negative, motile, non-sporulating; rod shaped, facultative anaerobic bacterium and is one of the main inhabitants of the intestinal tract of most mammalian species, including humans and birds. As it is present in high number in intestine, *E. coli* are often used as marker organisms. Detecting and counting of *E. coli* is used as a reliable indicator of fecal contamination and indicates a possible presence of enteropathogenic and/or toxigenic microorganisms in foods and water
which constitute a public health hazard (Kaper et al., 2004 and Dilbaghi and Sharma, 2007). Pathogenic E. coli are classified into categories based on the production of virulence factors and on the clinical manifestations that they cause. In addition to the presence of E. coli denoting fecal pollution, the presence of virulence-related genes in E. coli strains refers to the pathogenicity of the isolates (Klie et al., 1997; Jayarao and Henning, 2001 and Holko et al., 2006). The protein intimin (encoded by the chromosomal gene eae), is responsible for the intimate attachment of the bacteria to intestinal epithelial cells (Law, 2000 and Gyles, 2007). iutA encoding aerobactin system is one of serum survival genes, that are specific virulence markers associated with extraintestinal infection. The explanation of their occurrence could be the possibility of udder infection (Altalhi and Hassan, 2009). Isolates with the aerobactin system have a growth advantage in low-iron conditions (Montgomerie et al., 1984) and, in comparison to the other major specialized siderophore, enterobactin, aerobactin is more effective.

Considering the above mentioned data, the present study thus was planned to assess the bacteriological quality of some types of animal source foods by investigating Escherichia coli, which may contaminate them, through the following topics:

1- Collection of raw milk, raw beef meat and raw poultry meat samples from different localities in Ismailia City.
2- Isolation of suspected Escherichia coli isolates which may be found in them.
3- Biochemical identification of the isolated Escherichia coli.
4- Serological identification of some isolated Escherichia coli strains.
5- Antibiogram study of the isolated Escherichia coli strains.
6- Detection of the eaeA (protein intimin), iutA (encoding aerobactin) and iss (increased serum survival protein) gene factors that may be expressed in the examined Escherichia coli serovars.

Material and methods:
1- Isolation and identification of E.coli strains:
Escherichia coli were isolated from a total number of 246 raw food samples of animal origin, (105) raw milk samples, (31) beef meat samples and (110) poultry meat samples, by cultivation into 1% peptone water as pre-enrichment media, Brilliant Green Bile 2% broth as enrichment media, then on MacConkey's agar and by sub-cultivation on Sorbitol MacConkey's agar, sheep blood agar and Eosin methylene blue agar (EMB) and wrer identified by biochemical tests. Ten E. coli isolates were serologically identified by using slide agglutination test. Antibiotic
susceptibility of the isolated *E. coli* was performed, using standard plate technique as recommended by Clinical laboratory Standard Institute (CLSI). Four strains of the serologically identified *E. coli* were submitted to molecular studies for detection of the eaeA (protein intimin), iutA (encoding aerobactin) and iss (increased serum survival protein) genes by using PCR technique.

2-Molecular studies on *E. coli* strain

2-1- Extraction of DNA, according to QIAamp DNA mini kit instructions

1- 20 μl QIAGEN protease were pipetted into the bottom of a 1.5 ml micro centrifuge tube, 200 μl of the bacterial broth culture and 200 μl buffer AL were added to the sample, then mixed by pulse vortexing for 15 seconds, then the mixture was incubated at 56°C for 10 min. After incubation, the 1.5 ml micro centrifuge tubes were centrifuged to remove drops from the inside of the lid.

2- 200 μl ethanol (96%) were added to the sample, and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5 ml micro centrifuge tube was briefly centrifuged to remove drops from the inside of the lid.

3- The mixture from step 2 was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min.

The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

4- The QIAamp mini spin column was carefully opened and 500 ml buffer AW1 was added without wetting the rim. The cap was closed, and centrifuged at 8000 rpm for 1 min. The QIAamp mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.

5- The QIAamp mini spin column was carefully opened and 500 ml buffer AW2 were added without wetting the rim. The cap was closed, and centrifuged at full speed for 3 minutes.

6- The QIAamp mini spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. Centrifugation at full speed for 1 min was done.

7- The QIAamp mini spin column was placed in a clean 1.5 ml micro centrifuge tube, and the collection tube containing the filtrate was discarded. The QIAamp mini spin column was carefully opened and 100 μl buffer AE were added. The QIAamp mini spin column was incubated at room temperature (15-25°C) for 1 minute, and then centrifuged at 8000 rpm for 1 minute.

2-2-Oligonucleotide primers sequences of *E. coli* genes:

2-3- Cycling conditions of the different primers during PCR of
the tested *E. coli* genes according to specific authors and Emerald Amp GT PCR mastermix (Takara) kit.

2-4- **DNA Molecular weight marker**

The ladder (**Gel Pilot 100 bp ladder**) (cat. no. 239035), supplied from QIAGEN (USA). Number of bands: 6, Size range: 100-600 bp.) was mixed gently by pipetting up and down. 6 μl of the required ladder were directly loaded.

2-5- **Agarose gel electrophoresis according to Sambrook et al., 1989, with modification**

Electrophoresis grade agarose (2 g) was prepared in 100 ml TBE 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 ethedium 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. Ten to fifteen μl of each PCR product samples, negative control and positive control 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.

### Table (1): Oligonucleotide primers sequences of *E. coli* genes:

<table>
<thead>
<tr>
<th><strong>Prime r</strong></th>
<th><strong>Sequence</strong></th>
<th><strong>Amplifie d product</strong></th>
<th><strong>Reference s</strong></th>
</tr>
</thead>
</table>
| *eaeA*      | Forward/ GACCCGGCACAAGCATAAGC  
Reverse/ CCACCTGCAGCAACAAGAGG | 384 bp | Wen-jie JIN et al., 2008 |
| *Iss*       | Forward/ ATGTTATTTTCTGCCGCTCTG  
Reverse/CTATTGTGAGCAATATACCC | 266 bp | Yaguchi et al., 2007 |
| *iutA*      | Forward/ GGCTGGACATGGGAACCTGG  
Reverse/CGTCGGAACGGGTAGAATCG | 300 bp | |
Table (2) Cycling conditions of the different primers of the E. coli genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>No. of cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>eaeA</td>
<td>94°C 5 min.</td>
<td>94°C 45 sec.</td>
<td>54°C 45 sec.</td>
<td>72°C 45 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>Iss</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>54°C 30 sec.</td>
<td>72°C 30 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
<tr>
<td>iutA</td>
<td>94°C 5 min.</td>
<td>94°C 30 sec.</td>
<td>63°C 30 sec.</td>
<td>72°C 30 sec.</td>
<td>35</td>
<td>72°C 10 min.</td>
</tr>
</tbody>
</table>

Results:
*Biochemical examination revealed identification of 64 E. coli strains at percentage of 26.01% of the examined samples (27 strains, at percentage of 25.71% of the examined milk samples, 8 strains, at percentage of 25.80% of the examined beef meat samples and 29 strains, at percentage of 26.36% of the examined poultry meat samples).

*Serological identification of (10) E. coli isolated from the examined samples revealed that they were belonged to O_{111} polyvalent 1; O_{44} polyvalent 2 (2 strains for each); O_{78} polyvalent 4; O_{146} polyvalent 2; O_{26} polyvalent 1; O_{119} polyvalent 1; O_{86} polyvalent 1 and O_{18} polyvalent 3 (1 strain for each).

*The antimicrobial sensitivity of the isolated E. coli showed high susceptible rate to Ciprofloxacin (CIP), (100%), followed by Cefoperazone (CFP), (75%); Enrofloxacin (ENR), (73.44%); Cefotaxime (CTX), (70.31%); Streptomycin (S), (48.44%) and Colistin sulphate (CT), (45.31%). On the other hand, all strains (100%) were resistant to Ampicillin (AMP), Metronidazole (MTZ), Amoxicillin (AML) and Vancomycin (VAS).

*The molecular studied resulted in:
- The protein intimin (encoded by eae gene) was found in E. coli serovars ; O_{18} polyvalent 3; O_{111} polyvalent 1 and O_{44} polyvalent 2, with a size of 384 base pairs, while the examined gene was not found in E. coli serovar, O_{146} polyvalent 2.
- The aerobactin iutA gene was present in the four examined E. coli serovars , (O_{18} polyvalent 3; O_{111} polyvalent 1; O_{111} polyvalent 1; O_{44} polyvalent 2, and O_{146} polyvalent 2), with a size of 300 base pairs.
- coli, O_{146} polyvalent 2, did not have the increased serum survival iss gene, while the other 3 serovars (O_{18} polyvalent 3; O_{111} polyvalent 1 and O_{44} polyvalent 2) had it, with a size of 266 base pairs.
Table (3): Results of biochemical identification and frequency of distribution of E. coli among the examined food samples

<table>
<thead>
<tr>
<th>Types of samples</th>
<th>No. of samples</th>
<th>No. of isolated E. coli</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td>105</td>
<td>27</td>
<td>25.71</td>
</tr>
<tr>
<td>Raw beef meat</td>
<td>31</td>
<td>8</td>
<td>25.80</td>
</tr>
<tr>
<td>Raw Poultry meat</td>
<td>110</td>
<td>29</td>
<td>26.36</td>
</tr>
<tr>
<td>Total</td>
<td>246</td>
<td>64</td>
<td>26.01</td>
</tr>
</tbody>
</table>

Table (4) Results of serological identification of the examined E. coli strains (10 strains)

<table>
<thead>
<tr>
<th>Monovalent</th>
<th>Polyvalent</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>O_{111}</td>
<td>polyvalent 1</td>
<td>2 strains</td>
</tr>
<tr>
<td>O_{44}</td>
<td>polyvalent 2</td>
<td>2 strains</td>
</tr>
<tr>
<td>O_{78}</td>
<td>polyvalent 4</td>
<td>1 strain</td>
</tr>
<tr>
<td>O_{146}</td>
<td>polyvalent 2</td>
<td>1 strain</td>
</tr>
<tr>
<td>O_{26}</td>
<td>polyvalent 1</td>
<td>1 strain</td>
</tr>
<tr>
<td>O_{119}</td>
<td>polyvalent 1</td>
<td>1 strain</td>
</tr>
<tr>
<td>O_{86}</td>
<td>Polyvalent 1</td>
<td>1 strain</td>
</tr>
<tr>
<td>O_{18}</td>
<td>polyvalent 3</td>
<td>1 strain</td>
</tr>
</tbody>
</table>

Table (5) Antimicrobial sensitivity test of the isolated Escherichia coli serovars from the examined samples

<table>
<thead>
<tr>
<th>Antimicrobial groups</th>
<th>Antibacterial Agent</th>
<th>Concentration</th>
<th>Antibiotic sensitivity pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>Beta Lactams</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillins</td>
<td>Aminopenicillins (Pen A)</td>
<td>Amonoxillin (AML)</td>
<td>10 ug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ampicillin (AMP)</td>
<td>10 ug</td>
</tr>
<tr>
<td></td>
<td>3rd generation Cephalosporins (C_{3}G)</td>
<td>Cefoperazone (CFP)</td>
<td>75 ug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cefotaxime (CTX)</td>
<td>30 ug</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin (S)</td>
<td>10 ug</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Colistin sulphate (CT)</td>
<td>10 ug</td>
<td>29</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Vancomycin (VAS)</td>
<td>30 ug</td>
<td>0</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Ciprofloxacine (CTP)</td>
<td>5 ug</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Enrofloxacine (EnR)</td>
<td>5 ug</td>
<td>47</td>
</tr>
<tr>
<td>Others</td>
<td>Metronidazole (MTZ)</td>
<td>5 ug</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure (1)** Agarose gel electrophoresis of amplified eaeA gene PCR product (384bp)

**Lane 1:** Positive control for eaeA gene.

**Lane 2, 3 & 5:** Positive strains for eaeA gene (384 bp).

**Lane 4:** One step ladder (600bp).

**Lane 6:** Negative strains for eaeA gene.

**Lane 7:** Negative control.

**Figure (2)** Agarose gel electrophoresis of amplified iutA gene PCR product (300 bp)

**Lane 1:** Positive control for iutA gene.

**Lane 2, 3, 5 and 6:** Positive strains for iutA gene (300 bp)

**Lane 4:** 100 bp ladder (100-600bp).

**Lane 7:** Negative control for iutA gene.
Figure (3) Agarose gel electrophoresis of amplified *iss* gene PCR product (266 bp)

**Lane1**: Positive control for *iss* gene.

**Lane2**: Negative strain for *iss* gene.

**Lane 3, 5 and 6**: Positive strains for *iss* gene (266 bp)

**Lane 4**: 100 bp ladder (100-600bp).

**Lane7**: Negative control

**Discussion**

Out of 105 raw milk samples, *Escherichia coli* could be detected in 27 samples, comprising 25.71% of the examined raw milk samples. Corresponding results were obtained by El-Jendy, (2004), who isolated *E*. 24% of the examined samples. Higher values were reported by Abd-Allah (2002), (66%) from raw market samples & (86%) from raw bulk milk samples. Sudershan & Ashwani (1996), recovered *E. coli* at lower level (10.19%) of 108 raw milk samples, collected from Hisar, India. The presumed route of *E. coli* contamination of raw milk is via fecal contamination during milking (Hussein & Sakuma, 2005). However, direct excretion of the organisms from the infected udder has also been reported (Lira et al., 2004). Moreover, *E. coli* is regarded as an indicator of poor hygiene and sanitary practices during milking and further handling, (Altalhi & Hassan, 2009).

For the examined raw meat samples, 8 isolates of *Escherichia coli*, at percentage of (25.80%) of the examined samples. Nearly similar result was given by Mona (2002), (24%). Higher values were obtained by Elwi (1994), (93.3%). Lowe records were given Neveen (2002), (10%). Opposite result was stated by Khalafalla (1996), who determined 30 samples of raw meats (10 each of brisket meat, chuck meat and cubes from hind quarter) and *E. coli* were failed to be detected from any of the examined samples.
**E. coli** and **Salmonella** species are two of bacterial genera that commonly infect meat while it is being processed, cut, packaged, transported, sold and handled. These organisms spoiling meat may infect the animal either while it is live “endogenous disease” or may contaminate the meat after its slaughter “exogenous disease” (Lawrie & Ledward, 2006).

Twenty nine isolates of *Escherichia coli* were obtained from the examined raw poultry meat samples, at percentage of 29.59% of the examined samples. Corresponding results were mentioned by Mona (2002), (30%) Higher values were shown by Rofiel (1999), (40%); Lower result was given by Ghada (1997), (20%). Serological identification of (10) representative *E. coli* strains, isolated from the examined samples, raw milk, raw beef meat and raw poultry meat, revealed that they were belonged to O$_{111}$ polyvalent 1, O$_{44}$ polyvalent 2 (2 strains for each), O$_{78}$ polyvalent 4, O$_{146}$ polyvalent 2, O$_{26}$ polyvalent 1, O$_{119}$ polyvalent 1, O$_{86}$ polyvalent 1 and O$_{18}$ polyvalent 3 (1 strain for each). Noha (2008) isolated *Escherichia coli* from slaughtered and apparently healthy chicken, which serologically identified as O$_{78}$, O$_{1}$, O$_{2}$, O$_{8}$, O$_{27}$ O$_{119}$, O$_{126}$, O$_{125}$, O$_{26}$, O$_{87}$, O$_{128}$ and O$_{146}$.

Enteropathogenic *Escherichia coli* (EPEC) traditionally has been reserved for the classic serovars: O$_{26}$, O$_{86}$, O$_{111}$, O$_{114}$, O$_{119}$, O$_{125}$, O$_{128}$, O$_{142}$ and O$_{158}$ and are a special group organisms associated with out-breaks of children diarrhea (Wolfang et al, 1992).

All strains (100%) showed resistance to Ampicillin (AMP), Amoxicillin (AML), (both are related to Beta lactams, Aminopenicillins); Vancomycin (VAS), (Glycopeptides) and Metronidazole (MTZ).

Some of this bacterial resistance related with R-factor that lead to trance multiple resistance of antibiotic at some time and that happens mostly in bacteria that cause diarrhea (Smith et al, 1973; Ryder et al, 1980 and Sur et al, 2003).

The protein intimin (encoded by *eae* gene) was expressed in *E. coli* serovars; O$_{18}$ polyvalent 3; O$_{111}$ polyvalent 1and O$_{44}$ polyvalent 2, with a size of 384 base pairs, while the examined gene was not expressed in *E. coli* serovar O$_{146}$ polyvalent 2. Jelacic et al (2003) analyzed, by polymerase chain reaction, 82 Shiga toxin–producing *Escherichia coli* (STEC) isolates and found that all *E. coli* O157:H7 contained *eae*.

The aerobactin *iutA* gene was expressed in the four examined *E. coli* serovars, (O$_{18}$ polyvalent 3; O$_{111}$ polyvalent 1; O$_{44}$ polyvalent 2, and O$_{146}$ polyvalent 2), with a size of 300 base pairs. Altalhi and Hassan (2009) screened *E. coli* strains, isolated from raw milk sources, for markers of extraintestinal pathogenic *E. coli*
(ExPEC) using PCR assays and suggested that *iutA* (11 strains) was one of the most frequent virulence markers. Systems to meet the bacterial need for iron during infection is one of factors that are probably important for the establishment of Enterohaemorrhagic *Escherichia coli* (EHEC) in the gut and add to the bacterial virulence (*Welinder-Olsson and Kaijser, 2005*).

*E. coli* O$_{146}$ polyvalent 2, did not react the increased serum survival gene *iss* positively with *iss* gene, while the other 3 serovars (O$_{18}$ polyvalent 3; O$_{111}$ polyvalent 1 and O$_{44}$ polyvalent 2) expressed it, with a size of 266 base pairs. examined the prevalence of the three *iss* types among 487 *E. coli* isolates and found that the *iss* type 3 geneS was found to occur at a high frequency among extraintestinal pathogenic *Escherichia coli* (ExPEC) isolates, irrespective of the host source and reported that the increased serum survival gene *iss* has long been recognized for its role in extraintestinal pathogenic *Escherichia coli* (ExPEC) virulence.

The findings of this study revealed that some raw foods of animal origin sold to the public in some areas in Ismailia City were contaminated with *E. coli*. The possible sources of these contaminants are due to the unhygienic manner of handling. This implies that these foods are viable source of various diseases. Some of these diseases could spread and acquire epidemic status which poses serious health hazards. It is believed that cooking processes and hygiene could greatly reduce the microbial load to harmless level (*Agnes, 1995*).

**References**


