MOLECULAR DETECTION OF SHIGA TOXIN (stx1 and stx2) AND INTIMIN (eaeA) GENES IN ESCHERICHIA COLI ISOLATED FROM FECAL SAMPLES OF CATTLE, SHEEP, AND HUMAN IN BASRAH GOVERNORATE

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ABSTRACT

The present study aims to isolate and identify Escherichia coli from fecal samples of farm animals and human, also, it aims to molecular detection of shigatoxin and intimin genes in isolates. A total of (264) fecal samples and swabs were collected from different parts of Basrah in the period extending from September 2018 to January 2019. These samples were composed of (85) samples from cows, (94) samples from human and (85) samples from sheep. Different techniques were used in this study to detect the presence of E. coli; these techniques included conventional microbiological assays and molecular techniques (amplification of uidA gene by using polymerase chain reaction).

The results of these techniques indicated 50 (18.9%) were E. coli from the tested samples. These isolates were subjected to PCR to detect Shiga toxins and intimin genes (stx1, stx2, and eaeA). The results of PCR confirmed all (50) isolates were harbor at least one virulence gene. Out of 50 isolates 20 (40%) carried stx2 gene alone, the percentages of the carrier were (66.7 %, 41.7% and 23.5%) from human, sheep and cattle samples, respectively. The genes (stx1 and stx2) were detected together in 9/50 (18%), represent (52.9%) of cattle isolates. The intimin gene (eaeA) alone was detected in 2/50 (4%), represent (11.8%) of cattle isolates. (28%) of isolates harbor (stx2 and eaeA) genes, the isolates belong to human and sheep isolates (33.3%) and (45.8%), respectively. Presence of the genes (stx1, stx2, and eaeA) were discovered in (10%) of isolates, (11.8%) of cattle and (12.5%) of sheep.
The isolates were resistant to ampicillin, tetracycline (92%, 74%), respectively. However, the isolates were susceptible to imipenem, gentamycin, chloramphenicol, ciprofloxacin, and cefotaxime with a ratio of 100%, 92%, 78%, 68%, and 58%, respectively.

**INTRODUCTION**

*Escherichia coli* is a Gram-negative, facultative anaerobe, non-sporulating rod within the family Enterobacteriaceae. It can ferment different sugars, but lactose fermentation (with the production of acid and gas) is a characteristic of the species (1).

Shiga toxin-producing *E. coli* (STEC) appears to be widespread in the gastrointestinal tracts of wild and domestic animals and, not surprisingly, meat and other animal products are the significant sources of human infections (2). STEC has emerged as a group of foodborne pathogens that can cause severe human disease, such as hemolytic uremic syndrome (HUS) (3). The STEC strains are characterized by their ability to produce either one or both of these cytotoxins, referred to as Stx1 [first described as Shiga-like toxin I], Stx2 or [first described as Shiga-like toxin II] (4).

The STEC strains were isolated from a variety of animals. However, cattle are considered the main reservoir (5). Other studies have indicated that small domestic ruminants, including sheep and goats, are also key reservoirs of STEC (6 and 7). Moreover (8), stated that, the STEC remains a significant cause of foodborne-related disease in humans.

This study aimed to isolate and identify *Escherichia coli* form fecal samples and swabs from Humans and domestic animals (cattle and sheep) also, molecular detection of *E. coli* isolates that carrying gene (stx1, stx2, and eaeA).

**MATERIALS AND METHODS**

-Samples collection

The fecal samples and swabs were collected from different parts of Basrah province. A total of 264 fecal samples and swabs were collected from Cattle, Sheep, and Human in the period extending from September 2018 to January 2019. Table (1)
Table (1): Source, type, and numbers of samples

| Source of samples | Types of sample | No. of samples |
|-------------------|-----------------|----------------|
|                   | Fecal sample    | Swabs          |                 |
| Cattle            | 80              | 5              | 85              |
| Human             | 10              | 84             | 94              |
| Sheep             | 75              | 10             | 85              |
| Total             | 165             | 99             | 264             |

**Microbiological techniques**

**Isolation and identification of bacteria**

The samples were treated according to (9). Briefly, all the collected samples were transported immediately to the laboratory of Veterinary college by using an icebox. In the laboratory, the samples were inoculated in nutrient broth and incubate at 37°C for overnight. The preincubated samples were subcultured on MacConkey’s agar (Micromedia / Iran) and again incubated at 37°C for overnight. Next day 2–3 rose pink colonies randomly picked and were subcultured on EMB agar (Himedia / India) followed by overnight incubation at 37°C. The colonies which observed as a metallic sheen, single colony were subject to Gram’s stain (10), oxidase test; indole test and methyl red ( M.R) and voges – proskauer (V.P) Tests (11), and Citrate utilization test using Simmons citrate agar (12).

**Molecular techniques**

The suspected isolates of *E. coli* by using conventional microbiological techniques were submitted to conformation by amplification of *uidA* gene by using PCR technique. Bacterial DNA was extracted according to the manufacturer of bacterial extraction kit (Genaid, Korea). The primers of *uidA* gene (housekeeping gene of *E. coli*) was designed by using online software (GenScript Online PCR Primers Designs Tool), the sequence of *E. coli* from NCBI, the accession No. (CU928164.2.)
Table (2): Sequence of primer for designed *uidA* with their manufacture

| Primers | Primer sequences (5’→3’) | Length | Product size | Source   | Manufacturer |
|---------|---------------------------|--------|--------------|----------|--------------|
| *uid A* | F: 5’- CGTTGAACTGCGTGATGCGG -3’ | 20     | 203 bp       | This study | Bioneer / Korea |
|          | R: 5’- ACTGTTCGCCCTTCACGTC-3’ | 20     |              |          |              |

The reaction mixture of PCR was prepared in a total volume of 50μl for *uidA* gene. 25μl of Green master mix (Promega / USA), 2μl of each oligonucleotide primer, 10μl of DNA template and 11μl of Nuclease-free water.

Table (3): Amplification conditions of the *ds.uid A* gene.

| Stage | Step         | Temperature | Time   | No. of cycle |
|-------|--------------|-------------|--------|--------------|
| I     | Initial denaturation | 94°C        | 3 min. | 1            |
| II    | Denaturation  | 94°C        | 1 min. |              |
|       | Annealing    | 56°C        | 40 sec.| 30           |
|       | Extension    | 72°C        | 1 min. |              |
| III   | Final extension | 72°C        | 3 min. | 1            |

-Antimicrobial Susceptibility Test-

The confirmed isolates of *E. coli* were subjected to antimicrobial susceptibility test. This test was done according to the method of (13). The antibiotic disks were from (Bioanalyse/ Turkey), including Ampicillin (10μg), Cefotaxime (30 μg), Chloramphenicol (30 μg), Ciprofloxacin (5 μg) Gentamycin (10 μg), Imipenem (10 μg) and Tetracycline (10 μg).

-Molecular detection of Shiga toxins (*stxa1* and *stxa2*) and intimin (*eae A*) genes
Oligonucleotide primers for PCR amplification:

Primers used for detection of virulence genes (*stx1*, *stx2*, and *eae A*) in *E. coli* isolated from fecal samples of farm animals and human were adopted from (14), Table (4).

Table (4): Sequences of primers for stx, *stx2* and *eae A* genes

| Primers | Primer sequences (5′ → 3′) | Length | Product size | Source | Manufacturer |
|---------|-----------------------------|--------|--------------|--------|--------------|
| *stx1*  | F: 5′- AAATCGCCATTCTGGAGCTTCTCCTTTCTC-3′
R: 5′- TGCCATTCTGGCAACTCCGATGCA-3′ | 25     | 366 bp       | (14)   | Momtaz et al., 2012 |
| *Stx2*  | F: 5′-CGATCGTCCTACCTCGTTGATCTCA-3′
R: 5′-GGATATTCTCCACCCACTCTGACACCC-3′ | 25     | 282 bp       |        | Bioneer / Korea |
| *eae A* | F: 5′-TGCGGCGACACAGGGGGGCG-3′
R: 5′-CGGTGCCGCCACCAGGATTC-3′ | 20     | 629 bp       |        | |

The Amplification conditions

Amplification conditions of the genes (*stx1* and *stx2*) were optimized and illustrated in the table (5). However, the optimized amplification conditions of *eae A* gene listed in the table (6).

Table (5): Optimized amplification conditions for *stx1* and *stx2* genes

| Stage | Step         | Temperature | Time   | No. of cycle |
|-------|--------------|-------------|--------|--------------|
| I     | Initial denaturation | 94°C        | 3 min. | 1            |
| II    | Denaturation  | 94°C        | 30 sec. | 34           |
|       | Annealing    | 56°C        | 45 sec. |              |
|       | Extension    | 72°C        | 1 min.  |              |
| III   | Final extension | 72°C        | 5 min. | 1            |
RESULTS

The total number of collected samples was 264; the samples included fecal samples and swabs from farm animals (cows, sheep) and human. The isolation rate of *E. coli* identified by using conventional microbiological and molecular techniques, as shows in Table (7). The suspected isolates were confirmed as *E. coli* via detection of *uid A* gene by using the PCR technique, the size of product 203 bps, Figure (1). Of 264 samples 53 (20%) were identified by using conventional biochemical tests, however, of 53 suspected isolates 50 (94%) were confirmed as *E. coli*.

Table (6): Optimized amplification conditions for *eae A* gene

| Stage | Step               | Temperature | Time   | No. of cycle |
|-------|--------------------|-------------|--------|--------------|
| I     | Initial denaturation | 94°C        | 3 min. | 1            |
| II    | Denaturation        | 94°C        | 1 min  | 34           |
|       | Annealing           | 57°C        | 45 sec.|
|       | Extension           | 72°C        | 1 min. |              |
| III   | Final extension     | 72°C        | 3 min. | 1            |

Table (7): Number of *E. coli* isolates which Identified by using conventional microbiological and molecular techniques.

| Source of samples | Total No. | Suspected Isolates by using conventional microbiological techniques | Confirmed isolates by using Molecular detection *ds. uidA* |
|-------------------|-----------|-----------------------------------------------------------------|---------------------------------------------------------|
|                   | No.      | %                  | No.        | %                                   |
| Cattle            | 85        | 17                 | 20         | 17                                   | 20                                      |
| Human             | 94        | 10                 | 10.6       | 9                                    | 9.6                                     |
| Sheep             | 85        | 26                 | 30.6       | 24                                   | 28.2                                    |
| Total             | 264       | 53                 | 20         | 50                                   | 18.9                                    |
Figure (1): Electropherogram of *uidA* amplification products.

The mixture was run on 1.5% agarose gel, stained with ethidium bromide. M: marker, and *ds. uidA* product size (203 bp).

Note: The sample in well No. 1 was negative control, and (2-11) were positive samples.

**Antimicrobial susceptibility testing**

The *E. coli* isolates were subjected to seven antimicrobials as in table (8).
Table (8): Antibiogram of 7 antimicrobials were tested against (50 isolates) of *Escherichia coli*.

| Antimicrobial susceptibility | Cattle | Humans | Sheep | Total |
|-----------------------------|--------|--------|-------|-------|
|                             | R | I | S | R | I | S | R | I | S | R | I | S | NO | % |
| Ampicillin (AM)             | 15 | 0 | 2 | 9 | 0 | 0 | 22 | 2 | 0 | 46 | 92 | 2 | 4 | 2 | 4 |
| Cefotaxime (CTX)            | 6 | 1 | 10 | 6 | 1 | 2 | 6 | 1 | 18 | 18 | 36 | 3 | 6 | 29 | 58 |
| Chloramphenicol (C)         | 5 | 0 | 12 | 2 | 0 | 7 | 4 | 0 | 20 | 11 | 22 | 0 | 0 | 39 | 78 |
| Ciprofloxacin (CIP)         | 6 | 2 | 9 | 0 | 0 | 9 | 5 | 3 | 16 | 11 | 22 | 5 | 10 | 34 | 68 |
| Gentamycin (Gn)             | 3 | 0 | 14 | 0 | 0 | 9 | 1 | 0 | 23 | 4 | 8 | 0 | 0 | 46 | 92 |
| Imipenem (IMP)              | 0 | 0 | 16 | 0 | 0 | 9 | 0 | 0 | 25 | 0 | 0 | 0 | 0 | 50 | 100 |
| Tetracycline (TE)           | 13 | 1 | 3 | 7 | 1 | 1 | 17 | 3 | 4 | 37 | 74 | 5 | 10 | 8 | 16 |

The isolates were resistant to ampicillin, tetracycline, cefotaxime, chloramphenicol, and ciprofloxacin in percentage (92%, 74%, 36%, 22%, and 22%), respectively. Whereas the isolates were susceptible to imipenem, gentamycin, chloramphenicol, ciprofloxacin, and cefotaxime with a ratio of 100%, 92%, 78%, 68%, and 58%, respectively, Table (8).

**Molecular detection of stx1, stx2 and eaeA genes**

All isolates of *E. coli* were submitted to molecular detection of genes (*stx1, stx2, and eaeA*) by using primers. Table (10), figures (2) and (3).
Table (10): Distribution of \textit{stx}1, \textit{stx}2 and \textit{eaeA} genes in \textit{E. coli} isolates from different sources

| Samples source | only \textit{stx}1 | only \textit{stx}2 | \textit{stx}1 & \textit{stx}2 | only \textit{eaeA} | \textit{eaeA} & \textit{stx}1 | \textit{eaeA} & \textit{stx}2 | \textit{eaeA}, \textit{stx}1 & \textit{stx}2 |
|----------------|------------------|------------------|-------------------|-----------------|-----------------|-----------------|------------------|
|                | No   | %    | No   | %    | No   | %    | No   | %    | No   | %    |
| Cattle         | 0    | 0    | 4    | 23.5 | 9    | 52.9 | 2    | 11.8 | 0    | 0    | 0    | 0    | 2    | 11.8 |
| Human          | 0    | 0    | 6    | 66.7 | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 3    | 33.3 | 0    | 0    |
| Sheep          | 0    | 0    | 10   | 41.7 | 0    | 0    | 0    | 0    | 0    | 0    | 0    | 11   | 45.8 | 3    | 12.5 |
| Total          | 0    | 0    | 20   | 40   | 9    | 18   | 2    | 4    | 0    | 0    | 0    | 14   | 28   | 5    | 10   |

The total isolates which confirmed as \textit{E. coli} were subjected to PCR detection of genes \textit{stx}1, \textit{stx}2 and \textit{eaeA}. Out of 50 isolates 20 (40\%) carried \textit{stx}2 gene alone, the percentages of the carrier were (66.7 \%, 41.7 and 23.5) from human, sheep and cattle samples, respectively. The genes (\textit{stx}1 and \textit{stx}2) were detected together in 9/50 (18\%) only in cattle samples 9/17 (52.9\%). The intimin gene (\textit{eaeA}) alone was detected in 2/50 (4\%), the gene found in cattle isolates 2/17(11.8\%). Of 50 isolates 14 (28\%) were found as a carrier of (\textit{stx}2 and \textit{eaeA}) genes, the isolates belong to human and sheep isolates, 3/9(33.3\%) and 11/24 (45.8\%), respectively. Presence of the three genes (\textit{stx}1, \textit{stx}2, and \textit{eaeA}) were discovered in 5/50 (10\%) isolates, composed of 2/17 (11.8\%) and 3/24 (12.5\%) in cattle and sheep isolates respectively.

Figure (2): Electropherograms of Shiga toxin (\textit{stx}1 and \textit{stx}2) genes amplification

The mixture was run on 1.5\% agarose gel stained with ethidium bromide. Lanes: M, Marker. 1,2 and 4 have both [\textit{stx}1(366 bp) and \textit{stx}2 (282 bp) genes] while 3,5,6 and 7 has the only \textit{stx}2 gene.
Figure (3): Electropherograms of Amplification of *eaeA*.

The mixture was run on 1.5% agarose gel stained with ethidium bromide. Lanes: M, Marker, 3-5 have *eaeA* (629 bp) genes, while 1,2 were negative control.

**DISCUSSION**

*Escherichia coli* is a part of healthy enteric microbiota in humans and different animals. The characteristics of phenotypic and genotypic allow the identification of *E. coli* infective strains or pathovars (15). A source of human infection for some diarrheagenic *E. coli* group (DEC) strains are animal feces, particularly those capable of producing Shiga-like toxin which have been termed as Shiga toxin-producing *E. coli* (STEC) and especially EHEC which constitute the frequent cause of severe hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (16).

**Isolation rates of *E. coli***

The conventional microbiological methods used for *E. coli* detection relies on the enrichment of the sample on nutrient broth, then differentiating on MacConkey agar followed by culturing on selective media Eosin methylene blue and submitting to biochemical confirmation and molecular method according to (9).

The net isolation rate *E. coli* from total number of samples was (18.9%). The isolation rate of *E. coli* of human samples was 9.6%. This result was lower than that, reported by (17), who found *E. coli* in (21.4%) of stool culture. The isolation rate of *E. coli* from cattle in this study was
(20%), this rate was higher than (10.9%) which reported by (18) in Basrah province. On the other hand, the isolation rate of E. coli from sheep was (28.2%).

**Antimicrobial susceptibility**

Antimicrobial agents have primarily been used to cure infectious diseases caused by bacteria. The use of antibiotics is a significant risk factor for extension of resistance to these agents (19). Multidrug resistance in *Escherichia coli* has become a worrying issue that is increasingly observed in human but also in veterinary medicine worldwide (20).

By using the disc diffusion method, 50 isolates of *Escherichia coli* were submitted to antimicrobial susceptibility test toward 7 antimicrobial agents. The results of the current study clarified that *E. coli* isolate resistant to Ampicillin (92%), and 100% susceptible to imipenem. Table (8), these results are in agreement with (21) who found that the resistance to ampicillin was 100% and all of *E. coli* isolates were susceptible to imipenem. The reported susceptibility to gentamycin in this study was 92 %; this result is higher than 66.9% recording by (22). From the obtained result, the rate of resistance to tetracycline was 74%; this result is lower than 84.76% reported by (23) and the rate of resistance for chloramphenicol in this study was 22 %, this result is lower than 33.3%, found by (24). Furthermore, in this study, the resistance against cefotaxime and ciprofloxacin were found (36%, 22%) respectively. These results are similar to (22), who found that the resistance against Cefotaxime and ciprofloxacin were (36.6% and 18.3%), respectively.

The slight differences in resistance may result from the source of isolates; also, the reason behind continuous increasing in resistance to these antibiotics may attribute to over usage of these antibiotics. Consuming antibiotics without prescription has been assumed as one of the causes of reducing bacterial sensitivity to the antibiotics (25), moreover, in Iraq, antibiotics can be easily obtained without a prescription. All of the previous reasons might be responsible for such a high prevalence of resistance.

**Molecular detection of stx1, stx2, and eaeA genes**

Shiga toxin-producing *Escherichia coli* (STEC) are globally known pathogens that cause diarrhea, hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in humans. STEC are predominately shed within the feces of healthy meat-producing animal species and aren't
considered to be pathogens of ruminant species except once infections occur in young (pre-weaned) animals (26).

For 50 E. coli, isolates were submitted to PCR targeting the characteristics of virulence genes (stx1, stx2, and eaeA). All isolates were potentially pathogen hence harbor at least one specific virulence trait. This study revealed that the detection rate of stx2 gene in cattle was 23.5%. This result is lower than 35% who found by (27), while the percentage of stx2 gene in human was 66.7%, this result was higher than 24% reported by (28). Moreover, the stx2 ratio in sheep was 41.7%; this result was much higher than 14% found by (29), Table (10). Furthermore, stx1, no isolates have harbored this gene alone, this result is in agreement with (30), who has not detected the gene (stx1) in a study in the USA.

For both stx1 and stx2 genes were detected together (52.9%) only in cattle. This result was similar to (27), who noted that stx1 plus stx2 genes were harbored by 45.8%. Some studies have suggested that strains which possessing only stx2 are potentially more virulent than strains harboring stx1 or even strains carrying both stx1 and stx2 (31). Moreover, in mouse models, Stx2 is 100 times more potent than Stx1 (32).

Regarding eaeA gene, it was detected in 4% of isolates. This result is in agreement with (33), who reported that eae A gene was 4.1% isolates. Moreover, the distribution of the stx2 and eaeA genes of human in this study was 33.3%; this result was higher than 14.3% reported by (34). While in sheep, the distribution of the stx2 and eaeA genes was 45.8%, this result disagreed with (35) who found 8.7% of fecal samples were positive for both genes.

In the present study, the presence of the three genes (stx1, stx2, and eaeA) in both cattle and sheep was similar in percentage 11.8%, 12.5%, respectively, this result was comparable to (36), who found the presence of three genes in cattle and sheep was (6.52% and 11.42), respectively, Table (10).

**Conclusions**

All E. coli isolates were potentially pathogen; hence, harboring specific virulence genes. The plurality of isolates were resistant to ampicillin followed by tetracycline, whereas, all the isolates were susceptible to imipenem followed by gentamycin.
الكشف الجزيئي لجينات تيفان الشيفا (eaeA) وجينات الانتميين (stx2 و stx1) في الإشريكية القولونية المعزولة من عينات البراز المأخوذة من الأبقار والأغنام والبشر في محافظة البصرة

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الخلاصة

تهدف الدراسة الحالية إلى عزل وتحديد الاشريكية القولونية المعزولة من براز الحيوانات الحقلية (الأبقار والأغنام) والبشر. وواضحة تهدف إلى الكشف الجزيئي لجينات تيفان الشيفا والانتميين في العزلات. إذ تم جمع (246) عينة عياناً ومسحات من امكاني مختلفة من مدينة البصرة للفترة من ايلول 2018-تاين الثاني 2019. العينات التي تم جمعها تكونت من (85) عينة من الأبقار، (64) عينة من الإنسان، و (58) عينة من الأغنام. تم استخدام تقنيات مختلفة للكشف عن وجود الاشريكية القولونية و هذه التقنيات شملت الاختبارات البيئولوجية التقليدية والتكنولوجيات الجزيئية والتي تضمن (تشخيص جين ب.)، باستخدام تقنية تفاعل البلمرة المتسلسل (E.coli). نتائج هذه التقنيات تشير إلى تحديد 50 (18.9%) عينة تم اعتبارها محتملة

العزلة إلى تفاعل البلمرة المتسلسل للكشف عن جينات تيفان الشيفا والانتميين (stx1, stx2 و eaeA). كنتيجة البلمرة اكتسب أن كل العزلات (100%) تمثل على الأقل جين ضرورة واحد. من بين 50 عزلة وجد 20 (40%) من العزلات تحمل جين فقط (41.2%, 17.5%, 4.5%%) من عينات الإنسان، الأغنام والأبقار، على التوالي

كما تم كشف جينات (stx1 and stx2) معاً بنسبة 18% (0.5%)، تمثل (5%) من عزلات الأبقار. ثم كشف جين (eaeA) لوحده بنسبة 50% (0.4%)، وتمثل 11.8% من عزلات الأبقار. (28%) من العزلات تمثل جينات (stx2, and eaeA)، هذه العزلات تأتي من عزلات الإنسان والأغنام (32.3%)، و (4.8%) من التوالي. تم كشف وجود الجينات (stx1, stx2 و eaeA) في 10% من العزلات، (1.8%) من الأبقار، (1.6%) من الأغنام، و (8.5%) من التوالي.

العزلات التي تم تكييفها بتفاعل البلمرة تم اختبارها باستخدام الحساسية الدوائية ضد 7 من المضادات الحيوية، وبيبت النتائج اعث على مقاومة كانت ضد الإاميبيدين والترتراسكين (69.4%) على التوالي، في حين كانت العزلات حساسة إلى الإاميبيدين، الجنتاميسين، الكلورامفينكول، سيرفولوكساسي و سيفوتاكسيمي بنسبة (100%, 97.8%, 98.2%, 88.2%, 95%) على التوالي.
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301
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