



Virulence Factors and Antimicrobial Resistance Patterns of Non-O157 Shiga Toxin-producing *Escherichia coli* Isolated from Different Sources at Sadat City

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Authors' contributions

This work was carried out in collaboration between both authors. Author MSAEE designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. Author MM managed the analyses of the study and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: A great concern directed to non-O157 Shiga toxin-producing *Escherichia coli* (STEC) serotypes due to their public health importance. Detecting the existence, antimicrobial profiles, and virulence repertoire of different STEC serotypes from animals essential for human food are important.

Study Design: This study aimed to investigate the presence of STEC in different hosts, the distribution pattern of *stx1*, *stx2*, *eaeA*, and *hlyA* genes encoding Shiga toxins 1 and 2, intimin, and enterohemolysin, respectively, and the antimicrobial resistance of the detected serotypes.

Results: A total of 75 samples were collected, 20 fecal samples from broilers, 15 fecal samples from ducks, 20 beef samples, and 20 human urine samples. *Escherichia coli* was detected at a rate

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of 60/75 (80%) distributed as; 17 (85%), 8 (53.3%), 15(75%), and 20 (100%) from broilers, ducks, human urine, and beef samples, respectively. There was a significant difference between the isolation rates of *E. coli* from different sources with $p < 0.05$. The prevalent serotypes were O78, O2:H6, O15:H2, and O26: H1. The frequency of *stx1* gene was 56/60 (93.3%), *stx2* gene was 55/60 (91.6%), the *eaeA* gene was 35/60 (58.3%), and *hlyA* gene was 26/60 (43.3%). The most effective antimicrobials were amoxicillin/clavulanic acid and ampicillin with the efficacy of 52/60 (86.6%) for each, while the efficacy of chloramphenicol and doxycycline was 5/60 (8.3%) and 44/60 (73.3%), respectively.

Conclusion: The high frequency of non-O157 Shiga toxin-Producing *Escherichia coli* from different samples at Sadat City, high prevalence of virulence factors and resistance to chloramphenicol and doxycycline will help in monitoring the distribution of virulent serotypes and contribute to the establishment of control measures to reduce the spread of infection.

Keywords: Non-O157 Shiga toxin-producing *E. coli*; virulence genes; antimicrobial resistance.

1. INTRODUCTION

The most important reservoirs of non-O157 STEC serotypes are cattle, sheep, goats, swine, birds, wild animals, and humans that can harbor these notorious pathogens in their alimentary tract [1,2,3,4]. The Shiga toxin-producing *Escherichia coli* (STEC) is a pathotype capable of producing Shiga toxins 1 or 2 or both. STEC has been reported to be serious enteric foodborne zoonotic pathogen causing human gastrointestinal out breaks that range from mild watery diarrhea to bloody diarrhea and hemorrhagic colitis (HC) and could be complicated with the hemolytic uremic syndrome (HUS) all over the world [5,6]. The infection with non-O157 STEC surpassed that of O157 during the year 2011 in the United States, as the recorded prevalence was 35.9% and 64.1%, respectively [7]. Furthermore, non-O157 STEC infections are responsible for the majority of total STEC infections in Canada, Australia, Latin America, and Europe [8,9]. The top six non-O157 serogroups implicated in serious infections are O26, O45, O103, O111, O121, and O145 [10]. Recently, the genetic relatedness between the non-O157 STEC serotypes from the retail meat, milk, and human serotypes in Egypt was found [11]. Shiga toxins are the most important virulence factors associated with STEC. In humans, the glycosphingolipid Gb3, a molecule that is mostly found in kidney epithelium and endothelium, as well as microvascular endothelial cells in intestinal lamina propria is the target of *stx*, which attachment leading to damage of intestinal epithelial cells and kidney resulting in HC and HUS [12]. Shiga toxins are classified into two major types, *stx1* and *stx2*, which are encoded by the *stx1* and *stx2* genes, respectively. The *stx1* and *stx2* show 55% to 60% of amino acid homology. There are three

genetic variants of *stx1* which were *stx1a*, *stx1c*, and *stx1d*, while the variants of *stx2* are *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2g* [13]. A single STEC strain may harbor one or more Shiga toxin-encoding genes (*stx*) in their genome [14,15]. The O26:H11 strains have been shifting from *stx1* only to *stx1* and *stx2* and now to *stx2* only and considered more virulent than the other O26 strains [16,17,18]. The *stx*-phages can be found with high densities in fecal samples of healthy human as well as polluted environments and foods with human and animal feces [19,20,21]. The *stx*-phages carry the *stx* gene that have the ability to lysogenize non-virulent bacterial strains and convert them into STEC. Hence, the *stx*-phages could introduce new phenotypic traits to non-pathogenic serotypes, such as antibiotic resistance [22]. As a further complication, loss and gain of *stx*-encoding phages have been observed in O26:H11 strains [17].

Another important virulence factor is the *in* timin, encoded by the *eae* gene on the locus of enterocyte effacement (LEE), which leads to the formation of attaching and effacing (A/E) lesions [23]. Furthermore, the enterohemolysin is another virulence determinant encoded by the *ehxA* gene which is present on a 60-MDa virulence plasmid and causes hemolysis of host red blood cells [24].

Little information is available on the molecular and phylogenetic properties of non-O157 STEC from various sources, and the role of animal reservoir in causing human non-O157 STEC infection remains unknown. The aim of the study was to identify some non-O157 STEC isolates from multiple sources including broilers, ducks, human urine, and beef from Sadat city at Monufia Governorate. Furthermore, screening of

some virulence genes and antimicrobial resistance profiles was performed.

2. MATERIALS AND METHODS

2.1 Sample Collection

The collection of 75 samples was performed, 20 fecal samples from broilers, 15 fecal samples from ducks, 20 urine samples from human, and 20 beef samples, respectively. The sample collection was carried out in a period from November 2017 to November 2018 from different broilers ducks farms, retail meat, and central hospital at Sadat City in Minufia, Egypt. The fecal and meat samples were collected in sterile plastic bags while the urine samples were collected in sterile tubes, the samples were labeled and kept on ice then transported within 30 min to the laboratory of the Department of Bacteriology, Mycology, and Immunology Faculty of Veterinary Medicine University of Sadat City.

2.2 *E. coli* Isolation and Identification

The collected samples were pre-enriched in nutrient broth and incubated for 24 hrs at 37°C, after the appearance of confirmed growth, a loopful was streaked onto Mac Conkey agar medium plates (MAC) that incubated aerobically for 24 hrs at 37°C. The pink Lactose fermenting colonies were picked up with a sterile loop transferred onto Eosin Methylene Blue (EMB) and incubated for 24 hrs at 37°C. Discrete colonies with metallic green sheen typical of *E. coli* were inoculated into semisolid medium by stabbing technique, incubated for 24 hrs at 37°C and kept at 4°C. These were used for further investigation for their morphological, biochemical, serological, and molecular characterization. The isolated *E. coli* strains upon specific media were confirmed at the species level using different biochemical tests as cytochrome oxidase, indole test, triple sugar iron agar, and urease [25,26]. The *Staphylococcus aureus* ATCC 29737 was used as a negative control and *E. coli* (O157:H7, *stx1*, *stx2*, *eaeA*, *hlyA*) ATCC 35150 was used as a positive control.

2.3 Serological Identification of *E. coli*

The sero typing of confirmed isolates was performed using a slid agglutination test with specific antisera to *E. coli* somatic "O" and

flagellar "H" antigens. The implemented antisera were purchased from Denka-Seiken (Japan). The technique was performed after the manufacturer's instructions at The Center of Food Analysis, University of Banha and Faculty of Veterinary Medicine.

2.4 Molecular Characterization of Virulence Genes Harbored by *E. coli* Serotypes

The stock *E. coli* serotypes on the semisolid medium were inoculated on MAC agar medium plates. After overnight incubation at 37°C, few selected colonies were picked with a sterile toothpick for DNA extraction using the QI Aamp Miniprep kit and performed according to the manufacturer's instructions. All the *E. coli* serotypes obtained were examined for the presence of virulence genes using primers targeting *stx1*, *stx2*, *eaeA*, and *hlyA* (Table 1).

2.5 Antimicrobial Susceptibility Patterns

The confirmed serotypes were cultivated on Mueller-Hinton (Oxoid) broth for 18 hrs at 37°C until the bacterial density was adjusted to 1.5×10^8 /1 ml (using McFarland tube 0.5). Then, 1 ml from each tube was spread on Mueller-Hinton agar (Oxoid) plates. The Kirby-Bauer disk diffusion technique was performed using the following antimicrobial disks: ampicillin (AM) 10 µg, amoxicillin/clavulanic acid (AMC) 30 µg, chloramphenicol (C) 30 µg, and doxycycline (DO) 20 µg (Oxoid). The inhibition zones were measured after incubation for 24 hrs at 37°C, and the results recorded as (sensitive and resistant) and interpreted according to Clinical Laboratory Standards Institute [28].

2.6 Statistical Analysis

The STEC rates of isolation, the prevalence of the obtained serotypes and the sensitivity and resistance of isolates to antimicrobials are presented as percentages (%). The significance of difference between STEC isolation rates from different sources, between the serotypes, efficacy of different antimicrobials, and distribution patterns of virulence genes was determined using the Fisher's exact test and the Z-test in R statistical software at the statistical significance of $P < 0.05$.

Table 1. The primers used in PCR for detection of virulence genes in *E. coli*

| Primer | Oligonucleotide sequence (5' → 3') | Product size (bp) | References |
|----------|------------------------------------|-------------------|------------|
| Stx1(F) | 5' ACACTGGATGATCTCAGTGG '3 | | [27] |
| Stx1(R) | 5' CTGAATCCCCCTCCATTATG '3 | 614 | |
| Stx2(F) | 5' CCATGACAACGGACAGCAGTT '3 | | |
| Stx2 (R) | 5' CCTGTCAACTGAGCAGCACTTTG '3 | 779 | |
| eaeA (F) | 5' GTGGCGAATACTGGCGAGACT '3 | | |
| eaeA (R) | 5' CCCCATTCTTTTTACCGTCG '3 | 890 | |
| hlyA(F) | 5' ACGATGTGGTTTATTCTGGA '3 | | |
| hlyA (R) | 5' CTTACGTGACCATACATAT '3 | 165 | |

3. RESULTS

3.1 Prevalence of *E. coli*

A total of 75 samples collected were subjected to isolation and biochemical identification procedure using specific media. *Escherichia coli* was detected at a rate of 60/75 (80%) which was distributed as follows; 17/20 (85%), 8/15 (53.3%), 15/20 (75%), and 20/20 (100%) from broilers, ducks, urine and beef samples, respectively. Although the isolation and identification confirmed the existence of other enterobacterial agents such as *Klebsiella pneumoniae* from broilers, *Serratia liquefaciens* from ducks, *Enterobacter aerogenes*, *Providencia rettgeri*, and *Hafnia* species from human. There was a significant difference between these bacterial agents and the existence rates of *E. coli* serotypes with $p < 0.05$ (Table 2).

3.2 Prevalence of *E. coli* Serotypes and Antimicrobial Susceptibility

The most prevalent serotypes were O78, O2: H6, O15:H2, and O26:H11. The serotype O78 was the most prevalent from seventeen broilers fecal samples with a rate of 15/17 (85.0%). It showed pronounced resistance to chloramphenicol, 17/17 (100%) followed by resistance to doxycycline, 3/17 (17.6%). From the fecal samples of four duck, the serotype O78 was recovered with a rate of 4/8 (50%) and it was resistant to chloramphenicol and doxycycline with rates of 3/4 (75%) and 1/4 (25%), respectively. Furthermore, O2: H6 was isolated from fecal samples of the 4 duck with a rate of 4 (6.7%) it showed a pronounced resistance to chloramphenicol 4/4 (100%) and doxycycline 2/4 (50%). Additionally, O15:H2 obtained from fifteen human urine samples with a rate of 15/20 (75%) expressed clear resistance to chloramphenicol 14/15 (93.3%) followed by

doxycycline 5/15 (33.3%). The serotype O26:H11 represented a rate of 17/20 (85%) all of them from beef samples with a pronounced resistance to chloramphenicol 17/20 (85%) followed by doxycycline 5/20 (25%). In all, both ampicillin and amoxicillin/clavulanic acid exhibited the same efficacy on all serotypes as 13.4% of the serotypes were resistant to them, while doxycycline showed efficacy on 73.3% of serotypes. On the contrary to that, chloramphenicol showed the least efficacy on 8.3% serotypes. There were obvious significant differences between the efficacies of various antimicrobials on different serotypes with $p < 0.05$ (Table 3).

3.3 Molecular Characterization of Virulence Factors of Different *E. coli* Serotypes

The detection of virulence genes reveals that, *stx1* represented 50/60 (93.3%), *stx2* represented 55/60 (91.7%), *eaeA* represented 35/60 (58.3%), and *hlyA* represented 26/60 (43.3%). There was a significant difference between the distribution pattern of *stx1* and other virulence genes with $p < 0.05$. The O78 serotypes from broilers harbored *stx1* with a rate of 17/17 (100%), *stx2* with a frequency of 15/17 (88.2%), *eaeA* with a percentage of 12/17 (70.5%), *hlyA* with a frequency of 6/17 (35.2%). The four O78 serotypes from ducks contained *stx1* and *stx2*, 3/4 (75%) contained *eaeA*, and 1/4 (25%) contained *hlyA* gene. For the four O2:H6 from ducks, all of them contained *stx1* and *stx2* and the existence of *eaeA* and *hlyA* represented 50% for each. Concerning the fifteen O15:H2 serotypes from human urine 13/15 (86.6%) contained *stx1*, 14/15 (93.3%) harbored *stx2*, 10/15 (66.66%) contained *eaeA*, and 8/15 (53.3%) exhibited the *hlyA*. Concerning the twenty serotypes of O26:H11, the *stx1* and *stx2* represented 18/20 (90%) for each, the *eaeA* represented 8/20 (40%), and the *hlyA* represented 9/20 (45%) (Table 4 and Fig. 1).

Table 2. Results of collected samples and identified bacterial agents

| Source of samples | Type of samples | No. of samples | <i>E. coli</i> | | Other bacterial agents | | | | | | |
|-------------------|-----------------|----------------|----------------|------------------|------------------------------|------------------------------|-------------------------------|---------------------------------|-----------------------------|-----------------------|-------|
| | | | No. | Serotypes | <i>Klebsiella pneumoniae</i> | <i>Serratia liquefaciens</i> | <i>Enterobacter aerogenes</i> | <i>Enterobacter agglomerans</i> | <i>Providencia rettgeri</i> | <i>Hafnia species</i> | |
| Broilers | feces | 20 | 17 (85%) | O78 | 3(15%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) |
| Ducks | feces | 15 | 8(53.3%) | O78 (4)O2:H6 (4) | 0(0%) | 1(7%) | 6(40%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) |
| Human | Urine | 20 | 15 (75%) | O15:H2 | 0(0%) | 0(0%) | 2(10%) | 1(5%) | 1(5%) | 1(5%) | 1(5%) |
| Beef | meat | 20 | 20 (100%) | O26:H11 | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) | 0(0%) |

Table 3. Results of phenotypic antimicrobial susceptibility testing of different *E. coli* serotypes

| <i>E. coli</i> | Types of antimicrobials | | | | | | | |
|----------------|-------------------------|--------------|---------------------|---------------|------------------|---------------|-----------------------------------|--------------|
| | Ampicillin (AM) | | Chloramphenicol (C) | | Doxycycline (DO) | | Amoxycillin-clavulanic acid (AMC) | |
| | Sensitive | Resistant | Sensitive | Resistant | Sensitive | Resistant | Sensitive | Resistant |
| O78 (17) | 15 (88.2%) | 2 (11.7%) | 0 (0%) | 17 (100%) | 14 (82.%) | 3 (17.6%) | 16 (94.%) | 1 (5.8%) |
| O78 (4) | 4 (100%) | 0 (0%) | 1 (25%) | 3 (75%) | 3 (75%) | 1 (25%) | 3 (75%) | 1 (25%) |
| O2:H6 (4) | 3 (75%) | 1 (25%) | 0 (0%) | 4 (100%) | 2 (50%) | 2 (50%) | 4 (100%) | 0 (0%) |
| O15:H2 (15) | 13 (86.6%) | 2 (13.3%) | 1 (6.6%) | 14 (93.3%) | 10 (66.6%) | 5 (33.3%) | 14 (93.3%) | 1 (6.6%) |
| O26:H11 (20) | 17 (85%) | 3 (15%) | 3 (15%) | 17 (85%) | 15 (75%) | 5 (25%) | 16 (80%) | 4 (20%) |
| Total | 52/60 (86.6%) | 8/60 (18.3%) | 5/60 (8.3%) | 55/60 (91.7%) | 44/60 (73.3%) | 16/60 (26.7%) | 52/60 (86.6%) | 8/60 (18.3%) |

Table 4. Distribution of virulence factors of *E. coli* serotypes

| Origin of samples | Serotype | Virulence factors | | | |
|-------------------|--------------|-------------------|--------------|--------------|--------------|
| | | <i>stx1</i> | <i>stx2</i> | <i>eae</i> | <i>hlyA</i> |
| Broilers | O78 (17) | 17(100%) | 15(88.2%) | 12(70.5%) | 6(35.2%) |
| Ducks | O78 (4) | 4(100%) | 4(100%) | 3(75%) | 1(25%) |
| | O2:H6 (4) | 4(100%) | 4(100%) | 2(50%) | 2(50%) |
| Human | O15:H2 (15) | 13(68.6%) | 14(93.3%) | 10(66.66%) | 8(53.3%) |
| Beef | O26:H11 (20) | 18(90%) | 18(90%) | 8(40%) | 9(45%) |
| Total | 60 | 56/60(93.3%) | 55/60(91.7%) | 35/60(58.3%) | 26/60(43.3%) |

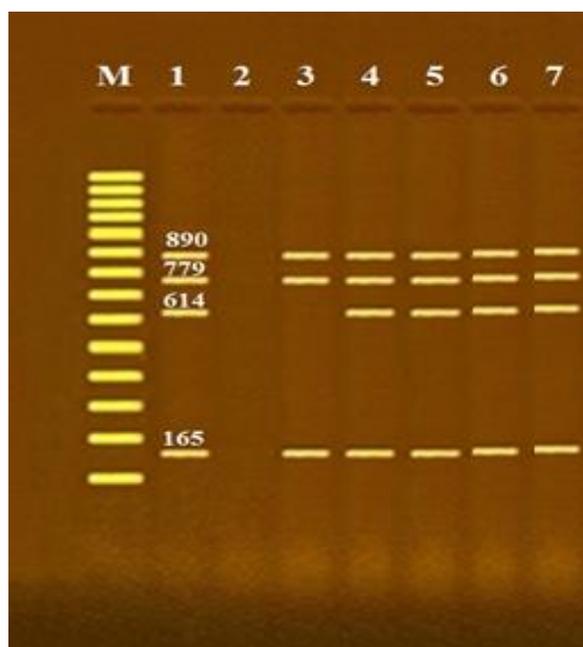


Fig. 1. Agarose gel electrophoresis of multiplex PCR of *stx1* (614 bp), *stx2*(779 bp), *eaeA*(890 bp) and *hlyA* (165 bp) genes for characterization of *E. coli*

Lane M: 100 bp ladder as molecular size DNA marker, Lane 1: Control positive *E. coli* for *stx1*, *stx2*, *eaeA* and *hlyA* genes, Lane 2: Control negative, Lane 3 (O78 from broilers): Positive strains for *stx2*, *eaeA* and *hlyA* genes, Lanes 4, 5, 6, and 7 (O78 from ducks, O2:H6 from ducks, O15:H2 from human, and O26:H11 from beef): Positive strains for *stx1*, *stx2*, *eaeA* and *hlyA* genes

4. DISCUSSION

The non-O157 Shiga toxin-producing *E. coli* serotypes are widely prevalent in different species and cause serious animal and human infections [1,2,3,4,7]. *E. coli* is the most notorious bacterial agent causes drastic losses in poultry and ducks farms. The results of isolation of STEC 17 (85%) from broilers and ducks agree with Parreira and Gyles [29] who reported a high proportion of STEC from chickens and possessed *stx* genes and these birds were also considered to be the reservoirs of STEC. Concerning the isolation from ducks, these results agree with Grossmann

et al. [30], however, the gained results disagree with Kobayashi et al. [31], who did not observe STEC in fecal samples from 199 broiler chickens in Finland. Also these results disagree with Schroeder et al. [32] who confirmed the absence of STEC in chicken, and Wani et al. [33] who could not isolate STEC from retail chicken and turkey obtained from Washington, DC, USA. The existence of O78 as the most prevalent serotype from broilers and ducks agree with Nataro and Kaper [34] who suggested that *E. coli* O78 is the most well-known serotype. The *E. coli* O15:H2 serotype was found in 15/20 (75%) of the human patients suffering from urinary tract infection (UTI) this result nearly similar to Flores-Mireles

et al. [35] who reported that uro pathogenic *E. coli* is the main cause of community-acquired UTIs (about 80- 90%). The existence of *E. coli* O15:H2 in UTIs agree with Abeet al. [36] and Yamamoto [37] who stated that *E. coli* O15, O16, O18, O21, O22, O25, and O75 serogroups are preferentially associated with UTI in human. The prevalence rate of *E. coli* isolates from beef samples was 20/20 (100%) and the serotype *E. coli* O26:H11 was highly prevalent. These results agree with Ethelberg et al. [38]. The Shiga toxins enter the host cells via a macropino some, the protein (A subunit) cleaves a specific adenine nucleobase from the 28S RNA of the 60S subunit of the ribosome, thereby preventing protein synthesis [39,40]. The *eaeA* gene encodes intimin which is an important virulence factor enable STEC to bind to their receptors on the intestinal cells and causes effacing lesions on intestinal epithelia [41]. The enterohemolysin is an intracellular toxin produced during the log phase of growth of *E. coli*, with a maximal intracellular accumulation in the late log phase, it is one of the cell-damaging protein toxins [42]. The virulence genes were harbored by most serotypes and *stx1* represented 50/60 (93.3%), *stx2* represented 55/60 (91.7%), *eaeA* represented 35/60 (58.3%), and *hlyA* represented 26/60 (43.3%). Both the *stx1* and *stx2* were highly prevalent, and that was similar to Elsayed et al. [27], although the distribution pattern of *eaeA* was nearly similar to Elsayed et al. [27], the existence rate of *hlyA* was lower than their findings.

Concerning the antimicrobial susceptibility testing, both Ampicillin and Amoxicillin/clavulanic acid exhibited the same efficacy on all serotypes as 86.6% of the tested serotypes were sensitive to them, while Doxycycline showed efficacy on 73.3% of serotypes. On the contrary to that, Chloramphenicol showed the lowest efficacy of 8.3%. These results come in contradiction with Elsayed et al. [27] who confirmed that STEC serotypes were highly sensitive to Chloramphenicol and Doxycycline which efficacy surpassed that of Ampicillin and Amoxicillin/clavulanic acid.

5. CONCLUSION

Different hosts as broilers, ducks, human, and beef represented vital reservoirs of Shiga toxin-producing *E. coli*, most of the obtained serotypes harbored many virulence genes and showed variable susceptibilities to used antimicrobials that considered crucial for public health. Hence,

the molecular typing and continuous monitoring of antimicrobial resistance could be beneficial for developing successful control strategies against STEC and for the formulation of new antimicrobials which decrease the possibility of antimicrobial resistance.

CONSENT AND ETHICAL APPROVAL

This study agrees with the U.S. Government rules for the employment and nursing of animals intended for experimental, training, and research objectives. The humane samples were collected after informed consent of the infected cases. The study design was accepted by the Faculty Committee for Animal Care and Use, University of Sadat City.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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