

Detection and Molecular characterization of Shiga-toxin producing *Escherichia coli* in fecal samples from dairy cattle of Northern Egypt

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Abstract

Objective: *Escherichia coli* that produce the Shiga toxin (STEC) has become one of the most significant foodborne pathogens linked to serious illnesses. Our study was aimed to detect the existence, antimicrobial profiles, and virulence repertoire of different STEC serotypes from fecal samples of dairy cattle.

Study design: We collected 19 fecal samples (12 diarrheic and 7 non-diarrheic) from dairy cattle. *E. coli* isolation and identification were performed. Shiga toxins genes were detected and the antimicrobial resistance was investigated.

Results: *E. coli* was detected at the rate of 13/19 (68.4%) from the fecal samples. The frequency of *Stx2a* represented 12/13 (92.3%), *Stx2* represented 3/13 (23.14%), and *Stx1d*, *Stx2f*, *Stx2g*, and *eae* represented 2/13 (15.4%). While the virulence genes *Stx1*, *Stx1c*, *Stx2c*, *Stx2d*, *Stx2e*, and *ehxA* represented 0/13 (0%). The most effective antimicrobials were tulathromycin 30 (53.84%) followed by ceftiofur 30 and marbofloxacin 5 (46.16%), amikacin (15.38%), and the least resistance was recorded for nalidixic acid and oxytetracycline (7.70%). The resistant antibiotics with percentages reached to 100% were amoxicillin/ clavulanic acid, ceftriaxone, ciprofloxacin, doxycycline, erythromycin, gentamicin, linezolid, nitrofurantoin, penicillin G, streptomycin, trimethoprim/sulfamethoxazole, and vancomycin followed by chloramphenicol, nalidixic acid, oxytetracycline, and tyrosine 30 (92.30%), amikacin and ampicillin (84.62%), ceftiofur 30 (23.07%), marbofloxacin 5 (15.38%) and tulathromycin 30 (7.70%).

Tyrosine is an amino acid – :[2HK]Comment
Tylosin is the antibiotic

Comment [HK3]: Tulathromycin - spelling

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Conclusions: The findings demonstrated that *E. coli* that are resistant to numerous groups of antimicrobials spread through feces and may pose a risk to human and animal health.

Keywords: Dairy cattle, Shiga-toxigenic *E. coli*, Virulence genes, Antimicrobial resistance,

1. Introduction

Escherichia coli (*E. coli*) is a common inhabitant of the human and animals' intestinal tract [1] and has been selected as a guard organism in worldwide antimicrobial

resistance (AMR) surveillance investigations[2,3]. Although ruminants are the main reservoir of STEC that contaminate environment and foods of animal and plant origins, STEC isolates were established in poultry, pet birds, wild birds and pigeons, dogs, and pigs [4,5]. *E. coli* is one of the most prevalent raw milk pollutants comes from a variety of sources, primarily animal feces. The constant presence of *E. coli* indicates fecal contamination, and the presence of additional enteric pathogens in raw milk puts consumers' health at risk[6]. Many dairy food products were contaminated with *E. coli* through the fecal-oral route as a result of poor hygienic measures in farms and the failure of pasteurization process[7].

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STEC infections are incriminated in hemolytic uremic syndrome and hemorrhagic colitis in human [8,9] and numerous food-borne epidemics[10]. In addition, STEC are associated with dysentery in cattle. Furthermore, they produce two types of Shiga toxins; *Stx1* and *Stx2*, and certain types of STEC have the ability to produce the intimin. The *E. coli* strains, which possess the *eaeA* gene and do not produce *stx1* and *stx2* genes, were defined as Enteropathogenic *E. coli*[11,12]. It is known that, *stx1a*, *stx1c*, and *stx1d* are the genetic variants of *stx1*, 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].

One of the greatest problems that affect the human and animal health all over the world is the antimicrobial resistance[16,17]. It is encoded genetically as a result of different causes including the unnecessary low dosage of antibiotics, feed additives, and preventive therapy in dairy animals [18,19]. Continuous shedding of the antibacterial resistant bacteria onto dairy farms and the surrounding environment occurred through feces, milk, or urine [20,21,22]. Moreover, it may transmit to humans through milk and meat consumption or manure land application as soil amendment for vegetables and crops for human consumption[23,24,25,26]. Antimicrobial resistance and virulence types in *E. coli* have been investigated on fecal or manure samples of dairy cattle in a variety of countries, including Egypt [1,27], Canada [28], USA [29], and Switzerland [30]. Therefore, this study was performed to determine the prevalence of *E. coli* in fecal samplings from dairy cattle in Egypt and determine their virulence factors and antibiotic sensitivity.

2. Materials and methods

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2.1. Samplings

A total of 19 fecal samples were collected from Egyptian dairy cattle. Twelve samples were from diarrheic feces and seven samples were from non-diarrheic feces. Sterile plastic bags were used for the fecal samples collection. The samples were labeled and kept on ice and transported to the Faculty of Veterinary Medicine, Sadat City University for examination in the laboratory of the Department of Bacteriology, Mycology, and Immunology.

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 MacConkey agar medium plates (MAC) and incubated at 37°C for 24 h. The pink Lactose fermenting colonies were picked up with a sterile loop transferred onto Eosin Methylene Blue (EMB) and incubated for 24 h at 37°C. Discrete colonies with metallic green sheen typical of *E. coli* were inoculated into the semisolid medium by stab technique, incubated for 24 h at 37°C, and kept at 4°C. The suspected colonies were used for further morphological, biochemical, and molecular characterization. The isolated *E. coli* strains upon specific media were confirmed at the species level using different biochemical tests such as cytochrome oxidase, indole test, triple sugar iron agar, and urease [31,32]. 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. Characterization of virulence genes harbored by *E. coli* serotypes

The preserved *E. coli* serotypes on the semisolid medium were inoculated on MAC agar medium plates. After overnight incubation at 37°C, a few selected colonies were picked with a sterile toothpick for DNA extraction using the QIAamp 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 primer targeting *stx1*, *stx2*, *eaeA*, and *hlyA*.

2.4. Antimicrobial susceptibility test

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)

with only 19 samples it will not be logical to generalize to entire Egypt as it has 27 governorates. So better, specify the area of study - e.g: Monufia Governorate of northern Egypt. **[7HK]Comment**

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plates. The disk diffusion technique was used to determine susceptibility to 21 antimicrobials in accordance with the Clinical and Laboratory Standards Institute [33] guidelines. The following antimicrobials were used: amikacin, amoxicillin/clavulanic acid, ampicillin, ceftiofur 30, ceftriaxone, chloramphenicol, ciprofloxacin, doxycycline, erythromycin, gentamycin, linezolid, marbofloxacin 5, Nalidixic acid, Nitrofurantoin, oxytetracycline, penicillin, streptomycin, trimethoprim/sulfamethoxazole, tulathromycin 30, tyrosine 30, and Vancomycin.

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2.5. Ethical Approval

The study was reviewed and approved by the Animal Use Ethics Committee of the Faculty of Veterinary Medicine, Sadat City University.

2.6. Statistical analysis

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3. Results

3.1. Prevalence of *E. coli* from dairy cattle fecal samples

A total of nineteen fecal samples from dairy cattle (12 diarrheic and 7 non-diarrheic) were exposed to isolation and biochemical identification procedures using specific media. Nine samples (75%) of the diarrheic samples were positive for *E. coli* while four samples of the non-diarrheic samples (57.14%) were positive. So, the total positive number and percentage for the isolation of *E. coli* was 13 (68.4%) as shown in Table 1.

Table 1: Results of isolation of *E. coli*

Samples	No. of collected samples	No. and percentage of isolates
Diarrheic Feces	12	9 (75%)
Non-diarrheic Feces	7	4 (57.14%)
Total Samples	19	13 (68.4%)

3.2. Percentage of virulence genes among the obtained isolates

The obtained results in Table 2 regarding the detection of virulence genes showed that *Stx2a* represented 12/13 (92.3%), *Stx2* represented 3/13 (23.14%), and *Stx1d*, *Stx2f*, *Stx2g*, and *ea* represented 2/13 (15.4%). While the virulence genes *Stx1*, *Stx1c*, *Stx2c*, *Stx2d*, *Stx2e*, and *ehxA* represented 0/13 (0%).

genes and proteins should be : [19HK] Comment appropriately abbreviated (https://wwwnc.cdc.gov/eid/page/scientific-nomenclature)

Table 2: Results of virulence genes of *E. coli*

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Isolate code	Source	Virulence factors											
		<i>Stx1</i>	<i>Stx1d</i>	<i>Stx1c</i>	<i>Stx2</i>	<i>Stx2a</i>	<i>Stx2c</i>	<i>Stx2d</i>	<i>Stx2e</i>	<i>Stx2f</i>	<i>Stx2g</i>	<i>eae</i>	<i>ehxA</i>
1	Diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	Diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
3	Diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4	Diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
5	Diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
6	Diarrheic feces	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
7	Diarrheic feces	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
8	Diarrheic feces	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
9	Diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve
10	Non-diarrheic feces	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
11	Non-diarrheic feces	-ve	+ve	-ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
12	Non-diarrheic feces	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
13	Non-diarrheic feces	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	-ve
Total		0/13 (0.0%)	2/13 (15.4%)	0/13 (0.0%)	3/13 (23.1%)	12/13 (92.3%)	0/13 (0.0%)	0/13 (0.0%)	0/13 (0.0%)	2/13 (15.4%)	2/13 (15.4%)	2/13 (15.4%)	0/13 (0.0%)

3.3. Antimicrobial susceptibility pattern of *E. coli* isolates

The positive *E. coli* samples were tested for antibiotic resistance and were applied antibiotic disks, amikacin, amoxicillin/clavulanic acid, ampicillin, ceftiofur 30, ceftriaxone, chloramphenicol, ciprofloxacin, doxycycline, erythromycin, gentamycin, linezolid, marbofloxacin 5, Nalidixic acid, Nitrofurantoin, oxytetracycline, penicillin, streptomycin, trimethoprim/sulfamethoxazole, tulathithromycin 30, tyrosine 30, and Vancomycin. The resistant antibiotics with

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percentages reached to 100% were amoxicillin/ clavulanic acid, ceftriaxone, ciprofloxacin, doxycycline, erythromycin, gentamicin, linezolid, nitrofurantoin, penicillin G, streptomycin, trimethoprim/sulfamethoxazole, and vancomycin followed by chloramphenicol, nalidixic acid, oxytetracycline, and tyrosine 30 (92.30%), amikacin and ampicillin (84.62%), ceftiofur 30 (23.07%), marbofloxacin 5 (15.38%) and tulathromycin 30 (7.70%). Antibiotics that revealed high antimicrobial activities with good effect for tulathromycin 30 (53.84%) followed by ceftiofur 30 and marbofloxacin 5 (46.16%), amikacin (15.38%), and the least sensitive was recorded for nalidixic acid and oxytetracycline (7.70%), (Table 3).

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Table 3: Results of antimicrobial susceptibility pattern of *E. coli* isolates

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No.		Amikacin	Amoxicillin/ clavulanic acid	Ampicillin	Cefixime 30	Ceftriaxone	Chloramphenicol	Ciprofloxacin	Doxycycline	Erythromycin	Gentamicin	Linezolid	Martifloxacin 5	Nalidixic acid	Nitrofurantoin	Oxytetracycline	Penicillin G	Streptomycin	Trimethoprim/sulfamethoxazole	Tulathromycin 30	Tylosin 30	Vancomycin
1	DF*	R	R	R	S	R	R	R	R	R	R	R	I	R	R	R	R	R	R	S	R	R
2	DF	R	R	I	I	R	R	R	R	R	R	R	S	R	R	R	R	R	R	I	R	R
3	DF	R	R	R	I	R	R	R	R	R	R	R	I	R	R	R	R	R	R	S	R	R
4	DF	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R
5	DF	R	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	R	R	I	R	R
6	DF	S	R	R	S	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	R
7	DF	R	R	R	I	R	I	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R
8	DF	R	R	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R
9	DF	R	R	R	S	R	R	R	R	R	R	R	I	S	R	R	R	R	R	I	R	R
10	NDF**	R	R	R	S	R	R	R	R	R	R	R	I	R	R	R	R	R	R	S	R	R
11	NDF	S	R	R	S	R	R	R	R	R	R	R	I	R	R	R	R	R	R	I	R	R
12	NDF	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R	R	S	R	R
13	NDF	R	R	R	I	R	R	R	R	R	R	R	S	R	R	R	R	R	R	I	R	R
S		2/13 15.38%	0/13 0%	0/13 0%	6/13 46.16%	0/13 0%	0/0 0%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	6/13 46.16%	1/13 7.70%	0/13 0%	1/13 7.70%	0/13 0%	0/13 0%	0/13 0%	7/13 53.84%	0/13 0%	0/13 0%
R		11/13 84.62%	13/13 100%	11/13 84.62%	3/13 23.07%	13/13 100%	12/13 92.30%	13/13 100%	13/13 100%	13/13 100%	13/13 100%	13/13 100%	2/13 15.38%	12/13 92.30%	13/13 100%	12/13 92.30%	13/13 100%	13/13 100%	13/13 100%	1/13 7.70%	12/13 92.30%	13/13 100%
I		0/13 0%	0/13 0%	2/13 15.38%	4/13 30.77%	0/13 0%	1/13 7.70%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	5/13 38.46%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	0/13 0%	5/13 38.46%	1/13 7.70%	0/13 0%

*DF: Diarrheic feces

**NDF: Non-diarrheic feces

4. Discussions

In this study, 68.4% (13/19) of fecal samples (diarrheic and non-diarrheic) from cattle were positive for *E. coli*. These results are nearly similar to the study that was performed in Egypt (63.6%) [34]. Furthermore, our findings are lower than what was reported in diarrheic calves in India (85.04%) [35]. However, a lower prevalence of *E. coli* was reported in previous investigations in Switzerland [36], Korea [37], Egypt [38], Argentina [39], and Ethiopia [40], with percentages of (5.5%), (22%), (28.8%), (30.1%), and (36.8%), respectively. These differences in the prevalence of *E. coli* may be attributed to different causes including, geography, hygienic measures for animals and the surrounding environment, health conditions of cattle and mixing of different ages of animals together [34,38,39,41]. Other researchers have similarly reported the isolation of *E. coli* from diarrheic calves in Egypt [27,42,43].

Concerning with the obtained findings of virulence genes, the *Stx2a* represented 12/13 (92.3%). The high *Stx2* percentage that is associated with clinical signs was concluded in the studies of [44] and [27] who stated that the *Stx2* gene was more prevalent than *Stx1* and that both were associated with *eae* gene in STEC strains. In contrast to our finding, the prevalence of virulence was *Stx1* and *eae* genes [45]. Moreover, the previous study performed by Elsayed et al., [46] reported that both the *stx1* and *stx2* genes were highly prevalent. In our study the *eae* genes were noticed in 15.4% of *E. coli* isolates, which is in accordance with Ishii et al., [47] but is in contrast to [48] and [49].

Regarding the results of antimicrobial resistance against *E. coli*, the isolated strains exhibited the maximum resistance (100%) against amoxicillin/ clavulanic acid, ceftriaxone, ciprofloxacin, doxycycline, erythromycin, gentamicin, linezolid, nitrofurantoin, penicillin G, streptomycin, trimethoprim/sulfamethoxazole, and vancomycin followed by chloramphenicol, nalidixic acid, oxytetracycline, and tyrosine 30 (92.30%), amikacin and ampicillin (84.62%), ceftiofur 30 (23.07%), marbofloxacin 5 (15.38%) and tulathromycin 30 (7.70%). In a previous study performed in Egypt [50] concluded that the most prevalent resistance antimicrobials were ampicillin (71.4%), amoxicillin (64.3%), trimethoprim/sulfamethoxazole (50%), and gentamicin (42.8%). Moreover, in Iranian investigation there was a maximum resistance of *E. coli* to lincomycin, penicillin, streptomycin, sulfamethoxazole, and tetracycline from diarrheic calves [51]. Furthermore, in Switzerland in 2022 data reported the antimicrobial pattern of resistance as ampicillin (48%),

genes and proteins should be appropriately abbreviated (https://wwwnc.cdc.gov/eid/page/scientific-nomenclature) :[34HK]Comment

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tetracycline(45%), and chloramphenicol (27%) [30].Notably, the present study reported that the *E. coli* isolates were sensitive to tulathithromycin 30, ceftiofur 30, marbofloxacin 5, amikacin, nalidixic acid, and oxytetracycline with the percentages of(53.84%), (46.16%),(46.16%),(15.38%),(7.70%), and(7.70%) respectively.However, Elsayed et al., [46]established that STEC serotypes were highly sensitive to chloramphenicol and doxycycline more than that of ampicillin and amoxicillin/clavulanic acid.Overall, our data of antimicrobial resistance in the study indicated the misuse of the veterinary drugs in the cattle farms and also the usage of the Egyptian paramedical people of these drugs.

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5- Conclusion

E. coli continues to be one of the major causes of severe economic losses all over the world. The Shiga toxin gene; *Stx2a*, is the most prevalent virulence genes associated with STEC followed by *Stx2*, *Stx1d*, *Stx2f*, *Stx2g*, and *eae*. The majority of the isolated *E. coli* have resistance pattern to amoxicillin/ clavulanic acid, ceftriaxone, ciprofloxacin, doxycycline, erythromycin, gentamicin, linezolid, nitrofurantoin, penicillin G, streptomycin, trimethoprim/sulfamethoxazole, vancomycin, chloramphenicol, nalidixic acid, oxytetracycline, and tyrosine 30, amikacin, and ampicillin. Moreover, tulathithromycin 30, ceftiofur 30, and marbofloxacin 5 are the most effective antimicrobial agents against the isolated *E. coli*. Our findings should inform veterinarians about the possible prognosis of *E. coli* infections in dairy cattle thereby reducing the unselective usage of antibiotics.

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