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Original Article *Zoonoses*

Characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle and their contacts

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ABSTRACT

Objective: To estimate the occurrence of Shiga toxin-producing *E. coli* (STEC) in cattle reared on farms versus those belonged to smallholders as well as their contact keepers.
***Design*:** Descriptive study.
***Samples*:** The study comprised 260 samples consisting of f ninety each of (*n* = 90) for each of cattle feces and milk samples that were originated either from smallholding cattle (n=30) or from farm cattle (n=60) alongside eighty stool specimens were collected from smallholders contacts (n=50) and farm workers (n=30).
Procedures: All samples were examined using conventional bacteriological and molecular techniques.
***Results*:** The overall occurrence of *E. coli* in animal samples was 25/180 with a percentage of 13.9. In cattle feces, it was 14.4% (13/90), whereas it was 13.3% (12/90) in milk samples. In human stool samples, *E. coli* i isolates were also identified in smallholders' contacts (14%, 7/50) and farm workers (16.7%, 5/30). The most prevalent serotypes obtained from farm cattle and their keepers were (O157 and O55) and from smallholders' cattle and their contact were (O111, O157, and O11). The PCR analysis showed that *stx2* was the most predominant genotypes followed by *stx1*. The recovered *E. coli* isolates showed high resistance to penicillin G (97.3%) and 81% (30/37) of the tested strains exhibited multidrug resistance.
Conclusion and clinical relevance: the presence of Shiga toxigenic and multiple drug-resistant strains of *E. coli* in the study area poses a high potential risk. Hence, strict hygienic measures should be followed to reduce the risk of STEC occurrence in smallholding cattle and those in commercial farms.

Keywords: **Shiga toxin-producing *E. coli*, cattle, milk, virulence genes**.

1. INTRODUCTION

Escherichia coli (*E. coli*) is one of the main gastrointestinal inhabitants in most mammalian species, including humans and birds. Most *E. coli* are commensal, but small proportions are potentially harmful and cause diseases worldwide **[1]**.Shiga toxins-producing *E. coli* (STEC) are a group of highly pathogenic strains known as enterohaemorrhagic *E. coli* (EHEC) or verotoxins-producing *E. coli* (VTEC) [2]. It is considered as one of the most emerging foodborne zoonotic bacteria causing wide range of various clinical symptoms including watery or bloody diarrhea, and potentially life-threatening syndromes such as hemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS) and acute renal failure [3]. Approximately 5% of STEC infection can develop HUS and the mortality can reach to 10% **[4].** Although *E. coli* O157 is the most serotype associated with food-borne outbreaks, other serotypes as O26, O55, O103, O111, O128, O91 and O145 which have been linked to cases and emerging outbreaks of HUS **[5].**

Cattle is considered as a natural reservoir for STEC and the pathogen can contaminate food products at any point along the production chain: during slaughtering, milking, storage or packaging **[6].** Strains of STEC are characterize by production of different virulence genes including shiga toxin (*stx1* and *stx2*) which inhibit the synthesis of host cell protein leading to cell death, beside the adherence factor, intimin, coded by *eae* gene which play a critical role in intestinal attachment **[5]**. An additional virulence factors that harbored by some strains of STEC is enterohaemorrhagic hemolysin which encoded by *hlyA* gene and associated with extra intestinal lesions and seems to affect several cells including erythrocytes, renal cells, lymphocytes and causing severe human illness **[7]**.

In Egypt, no restricted rules are outlined for the usage of antibiotics either for treatments of infected humans or interfacing animal diseases. The improper use of antibiotics can potentially cause the emergence of antimicrobial resistance foodborne zoonotic bacteria in animal-derived foods especially milk and meat that is usually associated with outbreaks globally **[8]**. The resistance of *E. coli* to wide ranges of antibacterial classes has been reported worldwide **[1]**, although the occurrence of multidrug resistance among *E. coli* strains is still a matter of concern. Despite the significance of STEC as emerging zoonotic illness, little is still known regarding their occurrence in apparently healthy smallholders and farm cattle as well as their contact persons.

The present study was, therefore, undertaken to evaluate the occurrence of STEC and their virulence associated genes as well as antimicrobial susceptibility in a linked study population and to correlate the isolated STEC strains from smallholder versus farm cattle and contact persons to estimate the potential zoonotic relatedness.

2. MATERIALS AND METHODS

2.1. Samples collection and preparation

The study comprised 260 samples consisting of cattle feces (*n* = 90) and milk samples (*n* = 90) that were originated either from smallholding cattle (n=30) or from farm cattle (n=60) alongside eighty stool specimens were collected from smallholder`s contacts (n=50) and farm workers (n=30). The study was conducted during 2018/2019 in Mansoura city, Dakahlia Governorate, Egypt. All the samples were collected under aseptic condition in sterile plastic cups and were transferred into individual sterile bags to be transported as soon as possible in insulated coolers to Hygiene and Zoonoses laboratory, Faculty of Veterinary Medicine, Mansoura University for bacteriological processing. The study follows the principles and specific ethical guidelines presented by Mansoura University also verbal consent was obtained from all owners prior to samples collection.

2.2. Isolation and Identification of E. coli

The isolation and identification of *E. coli* strains were performed according to standard methods ISO 7251:2005 **[9]**. Briefly one gram from each of animal fecal sample, human stool specimen and one mL of milk samples was added to 9 mL sterile Tryptone Soya Broth (TSB) **(Oxoid, CM 0192)** and were incubated at 37ºC for 18 hrs., th en an aliquot of 100µL was plated on Sorbitol MacConkey su pplemented with Cefixime (0.05mg/L) and potassium tellurite (2.5mg/L) (CT-SMAC) **(Oxoid CM0069),** then incubated at 37ºC for 24 hrs and were examined for the presence of suspected colonies. The suspected colonies were purified in Tryptone Soya Agar media **(Oxoid, CM 0981)** and were identified using different biochemical testes according to ISO 7251:2005 **[9].**

2.3. *Serotyping*

All the identified *E. coli* strains were serotyped by slide agglutination test using rapid diagnostic *E.coli* monovalent and polyvalent antisera sets according to Kok and others **[10]** in Food Analysis Center, Faculty of Veterinary Medicine, Benha University, Egypt.

2.4. DNA extraction

Genomic bacterial DNA was prepared by heating as previously described by Mohammed, and other reserachers **[5].** In brief five bacterial colonies of the same morphological shape were picked up and mixed with 100 µl of sterilized distilled water, then exposed to heat lysis in dry heat block at 95ºC for 15 minutes and centrifuged at 13000 rpm for 10 minutes. The supernatants were transferred to clean tubes and kept at - 20 ºC to use as DNA template.

2.5. Molecular Characterization of E. coli isolates

PCR reaction was performed in an individual reaction using thermal cycler (Biometra, Ltd, Kent, UK) to detect different virulence genes as *stx1*, *stx2*, *eaeA* and *hlyA*. The oligonucleotides primer sequences and their corresponding amplicon sizes were illustrated in (Table 1). The PCR reactions were carried out in a total volume of 20 µl consisting of 10µL of readymade PCR master mix (Fermentis, Biotech, Co.); 1 µL of each primer, 1µL of DNA template and completed to 20 µL with DNA free water. The amplification conditions were performed as described previously by Paton and Paton [11] (Table 1). An aliquot of each amplified product was run on a 1.5% agarose gel by electrophoresis then visualized and photographed by ultraviolet trans-illuminator.

2.7. PCR reaction for rfbE $_{O157}$ *and flic 7 genes*

Molecular confirmation of the presumptive *E. coli* O157:H7 isolates were performed using PCR assay to detect *rfbE* $_{O157}$ and *flic 7* genes. PCR reaction for both genes was carried out in a reaction mixture (25 µL) with the master mix (12.5µL), forward (1 µL) and reverse (1 µL) primers and template DNA (1 µL) and completed to 25µL with sterilized PCR water. The thermal cycling condition for amplification of *rfbE* $_{O157}$ was done according to Myataza and their colleagues **[12]**; while for *flic 7* was like that done by Sallam and others **[3].** The used amplification conditions were illustrated in (Table1). PCR products were run in 1.5% agarose gel for 40 minutes at 100 V then visualized and photographed by U.V light.

2.8. Susceptibility Testing

The susceptibility to kanamycin (30ug), ciprofloxacin (5ug), amikacin (30ug), penicillin G (10IU), erythromycin (15ug), cephalothin (30ug), nalidixic acid (30ug), gentamicin (10ug), cefotaxime (30ug), ampicillin (10ug), tetracycline (30ug), doxycycline (30ug), streptomycin (10ug) and sulphamethoxazol (25ug) was determined by the disk diffusion method as described in Clinical and Laboratory Standards Institute guidelines **[13].**

3. RESULTS

The overall occurrence of *E. coli* in animal samples was 25/180 with the percentage of 13.9, in cattle feces the recovery rate was 14.4% (13/90) of which 20% (6/30) and 11.7% (7/60) were recovered from cattle reared as smallholders and from those belonged to farm, respectively while in milk samples, the occurrence was 13.3% (12/90), including 16.7% (5/30) and 11.7% (7/60) was recognized as *E. coli* in smallholder and farm cattle milk, respectively. In human stool specimens, 14% (7/50) and (16.7%) 5/30 was identified as *E. coli* in smallholders contacts and farm workers, respectively (Table 2).

Serotyping of *E. coli* strains from smallholders cattle demonstrated that O119, O26, O157, O11, O111 and O113 were identified from cattle feces with the percentage of 12.5 for each; whereas O128, O157, O11, O55 and O146 were characterized in 20%, for each serotype in cattle milk. For the owners of smallholders, the following serotypes were identified: O84, O111, O146, O11, O157, O55 and O111 with

a percentage of 14.3%, for each (Table 3). For cattle reared in farm, the obtained results revealed that O146, O111, O55, O11 and O157 were identified with the percentage of 14.3% for each serotype in animal feces; whereas in milk the serotype were as follow: O26, O91, O121, O157, O55, O146 and O113 14.3%, for each. While in farm workers, the obtained strains were O128, O55, O11, O157 and O128 with the percentage of 20%, for each (Table 4). Molecular characterization of the identified *E. coli* strains from animals' sources demonstrated that all the examined virulence genes were detected among animals' samples with the percentage of 32%. (8/25) whereas, 24 % (6/25) for *stx2, eaeA, hlyA* and it was 3/25 (12%) for *stx1, eaeA* and *hlyA*. In human samples the predominant genes were *stx1*, *stx2*, *eaeA* and *hlyA* with the percentage of 25% (3/12)/ 16.7% (2/12) for each of (*stx2, eaeA, hlyA / stx1, eaeA, hlyA* and *stx1, stx2,* and *eaeA*). The recovered *E. coli* isolates showed high resistance to penicillin G (97.3%) followed by cephalothin and ampicillin (83.8%, per each), erythromycin (72.9%), however; most of the tested *E. coli* isolates showed susceptibility to gentamicin (91.9%), amikacin (89.2%), ciprofloxacin (86.5%), doxycycline, kanamycin (72.9%, per each) and sulphamethoxazol (67.5%), respectively whereas 81% (30/37) of the tested isolates exhibited multidrug resistance (Table 5).

Table 1. Oligonucleotide primers sequences used for amplification of virulence associated genes in STEC isolates.

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	PCR cycling (35 cycles) conditions	References
Stx1 (F)	**5′** ATAAATCGCCATTCGTTGACTAC **′3**	180		
Stx1 (R)	**5′** AGAACGCCCACTGAGATCATC **′3**			
Stx2(F)	**5′** GGCACTGTCTGAAACTGCTCC **′3**	225		
Stx2(R)	**5′** TCGCCAGTTATCTGACATTCTG **′3**			
eaeA (F)	**5′** GACCCGGCACAAGCATAAGC **′3**	384	95 °C for 5min	**Paton and Paton (1998)**
eaeA (R)	**5′** CCACCTGCAGCAACAAGAGG **′3**		58 °C for 30 s	
			72 °C for 30 s	
hlyA (F)	**5′**GCATCATCAAGCGTACGTTCC**′3**	530		
hlyA (R)	**5′**AATGAGCCAAGCTGGTTAAGCT**′3**			
rfbE$_{O157}$(F)	**5′** CTACAGGTGAAGGTGGAATGG**′3**	327	94 °C for 30 s	**Myataza et al. (2017)**
rfbE$_{O157}$(R)	**5′** ATTCCTCTCTTTCCTCTGCGG**′3**		60 °C for 90 s	
			72 °C for 90 s	
fliC7(F)	5′-CGGATGGCACAAGTCATTAATACC-3′	1758	98 °C for 10 s	**Sallam et al. (2013)**
fliC7(R)	5′-TTAACCCTGCAGCAGAGACAGAA-3′		58 °C for 30 s	
			68 °C for 90 s	

Table 2. Frequency distribution of *E. coli.*

	Samples	Examined	Positive	%
Cattle	Smallholder feces	30	6	20
	Farm feces	60	7	11.7
	Total	**90**	**13**	**14.4**
Milk	Smallholder milk	30	5	16.7
	Farm milk	60	7	11.7
	Total	**90**	**12**	**13.3**
Human	Smallholder contacts	50	7	14
	Farm workers	30	5	16.7
	Total	**80**	**12**	**15**
Total		**260**	**37**	**14.2**

4. DISCUSSION

Infection by STEC is becoming a potential health concern and is gained a special attention worldwide. In the present study, the occurrence of *E. coli* in cattle feces concurred with that given by Rehman and others **[14],** while being higher than those reported by [15, 16] and lower than those reported by others **[6, 17]**. For milk samples, our findings were consistent with previous studies **[6, 18]** who identified E coli in 19% and 17% *E. coli* in 19% and 17% of the examined milk samples, respectively. In contrast, high isolation rates (30, 66.6 and 38%) were reported by other researcher **[19,20,17]**. The presence of *E. coli* in human samples were in agreement with that previously mentioned by Awadallah et al**. [21]** who identified *E. coli* in 20% from the examined samples; while high rates of occurrence were given by **[19, 20].** Nevertheless, low rates of occurrence (2.9, 8.9, 7%) were reported by other researchers **[7, 22, 23]**. The difference in the isolation rate of *E. coli* in this study and other studies could be attributed to the differences in samples size, diversity of sampling and the methods used for isolation and characterization besides the lack of awareness, and poor personal hygiene.

In Egypt, nearly 70% of the livestock animals are reared by small farmers in which cattle are reared as domestic animal and account for 80-85% of the livestock population **[24]**. Our findings demonstrated that the most prevalent serotypes in smallholders and their contacts were O111, O157 and O11 (16.7%; 3/18, per each) and these were like

that previously reported by other researchers **[2, 6, 25, 26]**. The PCR analysis showed that the most predominant genotypes were *stx2* (15/18) 83.3%, *stx1* (13/18) 72% and *eaeA* genes (12/18) 66.7% and this agreed with that previously mentioned by several researchers **[6, 27, 28]** while low detection rates were reported by other researchers, Kalender **[29]** who identified *stx1* and *stx2* with the percentages of 18, 14, respectively in the examined cattle rectal swabs. Also, Ramadan and others **[2]** detected *stx1*, *stx2* and *eaeA* genes with the percentages of 27.8, 19.4 and 50, respectively in stool samples.

Table 3. Antibiograms, serotypes and molecular characterization of *E. coli* strains isolated from smallholders' animals and their contacts.

Isolate origin	Serotype	% of recovery	Positive genes	Resistance profile	Multidrug resistant index
Smallholder animal feces n=6	O119	16.7	*Stx1, stx2*	S, E, NA, P, CN, SXT, CF, T, AM	0.643
	O26	16.7	*Stx1, stx2, eaeA, hlyA*	S, E, NA, P, CN, SXT, CF, T, AM, K, CP, AK, DO	O.928
	O157	16.7	*Stx2, eaeA, hlyA*	E, P, CN, AM	0.285
	O11	16.7	*Stx1, eaeA, hlyA*	P, CN, AM, K	0.285
	O111	16.7	*Stx1, stx2, eaeA, hlyA*	S, E, NA, P, CN, SXT, CF, T, AM, DO	0.714
	O113	16.7	*Stx1, stx2, eaeA, hlyA*	P, AM, K	0.214
Smallholder animal milk n=5	O128	20	*Stx1*	S, E, NA, P, CN, SXT, CF, T, AM, K, CP, AK, DO, G	1
	O157	20	*Stx2, eaeA, hlyA*	E, P, CN, AM	0.285
	O11	20	*Stx1, stx2, eaeA, hlyA*	P, CN, AM, K	0.285
	O55	20	*Stx2, eaeA, hlyA*	S, E, P, CN, CF, T, AM, DO	0.571
	O146	20	*Stx1, stx2, hlyA*	P, CN, AM	0.214
Smallholder contacts n=7	O84	14.3	*Stx1*	S, E	0.143
	O111	14.3	*Stx2, hlyA*	S, E, NA, P, CN, SXT	0.428
	O146	14.3	*Stx1, stx2, eaeA*	P, CN, AM	0.214
	O11	14.3	*Stx1, stx2, eaeA*	P, CN, AM, K	0.285
	O157	14.3	*Stx1, stx2, eaeA, hlyA*	E, P, CN, AM, G	0.357
	O55	14.3	*Stx1, stx2, eaeA, hlyA*	E, P, CN, T, AM, DO	0.428
	O111	14.3	*Stx1, stx2, hlyA*	S, E, NA, P	0.285

Our findings demonstrated that O157 (26.3%) and O55 (15.8%) were the most prevalent serotypes obtained in farm cattle and their workers which were in harmony with that reported by different researchers **[17, 18, 30]**. On the other side, the main virulence genes determined in cattle reared in farm and their contacts were *stx2* 84.2% (16/19), *stx1* 68.4% (13/19) and 84.2% (16/19) for *eaeA* gene which were in agreement with that reported in other studies **[6, 7, 31]** they found that *stx2* was the predominant genotype in different examined sources while being disagreed with Abotalp and others **[17]** who found that *stx1* was the predominant genotype in the examined cattle feces.

The identified serotypes in the study were categorized as EHEC (O157, O111) which known to cause HC and HUS in human **[32]**, EPEC (O55) which responsible for infantile diarrhea **[33]** and ETEC (O11) was also identified and it was incriminated in severe cholera like syndrome **[34]**. Several previous researchers have reported that strains carrying *stx2* are potentially more virulent than those carrying *stx1* or even strains carrying both *stx1* and *stx2* are often implicated with HUS **[35, 36].** Other researchers have showed a strong association between the carriage of *eaeA* gene and the ability of STEC strains to cause severe human disease, particularly HUS **[35, 37]**.

In the present study, most *E. coli* strains provoked resistance to penicillin G followed by cephalothin, ampicillin, and erythromycin. These findings agreed with that given by Kalender **[29]** who found that all the tested isolates (*n*=22) which recovered from cattle rectal swabs in Turkey were resistant to pencillin and were sensetive to gentamicin. While in Egypt, Sheikh et al. **[31]** recorded that 74%, 60% and 54% of *E. coli* isolates which recovered from milk and milk products were sensetive to ciprofloxacin, gentamicin and nalidixic acid, respectively while 68%, 60% and 60% of the tested isolates were resistant to Amikacin, Ampicillin and kanamycin, respectively. Meanwhile, in a recent study, it was found that six strains of EHEC isolated from raw milk exhibited high resistance to erythromycin (100%), streptomycin (97.2%) and nalidixic acid (86%) while showed low resistance to amikacin (25%), ciprofloxacin (19.4%),

kanamycin (13.89%) and gentamicin (2.7%) **[26]**. Collectively, the high resistance of *E. coli* strains to the tested antimicrobials could be attributed to the excessive and uncontrolled usage of these antimicrobial agents in the treatment of various clinical entities under field condition.

The present study has its own limitation including unequal sample size collected from smallholder cattle and those raised in the commercial farm. Further studies are also needed to elucidate the potential ability of different STEC serotypes to form biofilm.

Conclusion

The presence of shiga toxigenic and multiple drug resistant strains of *E. coli* in the study area poses a high potential risk especially in the absence of strict hygienic practices and preventive measures. Hence, strict hygienic measures should be followed to reduce the risk of STEC occurrence in smallholding cattle and those in commercial farms and to avoid the dissemination of such pathogenic *E. coli* strains in food production chains

Table 5. Antimicrobial susceptibility of *E. coli* strains (n=37) isolated from different sources.

Antimicrobial agent	S		R	
	No	%	No	%
Streptomycin (S)	20	54	17	45.9
Erythromycin (E)	10	27	27	72.9
Nalidixic acid (NA)	23	62	14	37.8
Penicillin G (P)	1	2.7	36	97.3
Cephalothin (CN)	6	16.2	31	83.8
Sulphamethoxazol (SXT)	25	67.5	12	32.4
Cefotaxim (CF)	23	62	14	37.8
Tetracycline (T)	23	62	14	37.8
Ampicillin (AM)	6	16.2	31	83.3
Kanamycin (K)	27	72.9	10	27
Ciprofloxacin (CP)	32	86.5	5	13.5
Amikacin (AK)	33	89.2	4	10.8
Doxycycline (DO)	27	72.9	10	27
Gentamicin (G)	34	91.9	3	8

Table 4. Antibiograms, serotypes and molecular characterization of *E. coli* strains isolated from farm cattle and their workers.

Isolate origin	Serotype	% of recovery	Positive genes	Resistance profile	Multidrug resistant index
Feces n=7	O157	14.3	*Stx1, stx2, eaeA, hlyA*	S, E, NA, P, CN, SXT, CF, T	0.571
	O146	14.3	*Stx2*	S, E, NA, P, CN, SXT, CF	0.500
	O111	14.3	*Stx1, stx2, eaeA, hlyA*	S, E, NA, P, CN, SXT, CF, T, AM, K, CP, AK, DO, G	1
	O55	14.3	*Stx2, eaeA, hlyA*	S, E, P, CN, CF, T, AM, DO	0.571
	O11	14.3	*Stx2, eaeA, hlyA*	P, CN, AM, K	0.285
	O157	14.3	*Stx1, stx2, eaeA*	E, P, CN, AM	0.285
	O157	14.3	*Stx1, stx2, eaeA, hlyA*	E, P, CN, AM	0.285
Milk n= 7	O26	14.3	*Stx1, stx2, eaeA, hlyA*	S, E, NA, P, CN, SXT, CF, T, AM	0.643
	O91	14.3	*Stx1, stx2 hlyA*	S, E, NA, P, CN, SXT, CF, T, AM, K, CP	0.786
	O121	14.3	*Stx1, stx2*	S, E, NA, P	0.285
	O157	14.3	*Stx1, eaeA, hlyA, rfbE*$_{O157}$	E, P, CN, AM	0.285
	O55	14.3	*Stx1, stx2, eaeA*	S, E, P, CN, CF, T, AM, DO	0.571
	O146	14.3	*Stx1, stx2, eaeA*	P, CN, AM	0.214
	O113	14.3	*Stx2, eaeA, hlyA*	P, AM, K	0.214
Worker n=5	O128	20	*Stx2, eaeA, hlyA*	S, E, NA, P, CN, SXT, CF, T, AM, DO	0.714
	O55	20	*Stx1, stx2, eaeA, hlyA*	E, P, CN, AM	0.285
	O11	20	*Stx1, eaeA, hlyA*	P, AM, K	0.214
	O157	20	*Stx1, eaeA, hlyA, flic7*	E, P, CN, AM	0.285
	O128	20	*Stx2, eaeA, hlyA*	E, P, CF, AM S, E, NA, P, CN, SXT, CP, T, AK, DO	0.714

Conflict of interest

There is no conflict of interest.

Author contributions:

Shimaa El-Mahmoudy performed the lab work and wrote the first draft, Mayada Giwda designed the study, management and coordination responsibility for the research activity planning and execution, reviewing and editing the

article. Adel El-Gohary and Amro Mohamed revised the final version. All authors have read and approved the final version of the manuscript for publication.

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