



RESEARCH ARTICLE

Virulence Repertoire and Antimicrobial Resistance Profile of Shiga Toxin-Producing *E.coli* Isolated from Sheep and Goat Farms from Al-buhayra Egypt

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ABSTRACT

Shiga toxin-producing *E.coli* (STEC) evokes a paramount concern from the public health point of view. Many reports dealt with the characterization of STEC from large ruminants. This study aimed to investigate the presence of STEC in sheep and goats, distribution of *stx1*, *stx2*, *eaeA*, and *hlyA* genes encoding Shiga toxins, intimin, enterohemolysins, and the antimicrobial resistance index (MAR). A total of 170 samples collected from (diarrheic, apparently healthy, and milk samples) from sheep, goats, and bedding (136, 27 and 7) respectively. *E.coli* was detected at a rate of 71 (41.7%) distributed as 62 (44%) and 9 (31%) from sheep and goat, respectively. The prevalent serotypes were O111: H2, O26: H11, O103:H2, O55: H7, O86, O121: H7, O125: H21, and O124. The frequency of *stx1* gene was 13/15 (86.7%), *stx2* was 14/15 (93.3%), the *eaeA* gene was 8/15 (53.3%), and *hlyA* gene was 10/15 (66.7%). The most effective antimicrobials were Chloramphenicol, Doxycycline and Cephadrine. It was clear that 6/15 (40%) of the obtained serotypes exhibited MAR index ≤ 0.5 while 9/15 (60%) gave MAR index > 0.5 with a significant difference between them ($P < 0.05$). Hence, genotyping and antimicrobial resistance are pivotal epidemiological tools promoting felicitous control strategies against STEC serotypes.

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INTRODUCTION

Recently the *E.coli* classification is based upon pathotype detection. The pathotype that produces Shiga toxins (STEC) is characterised by its capacity to contain one of the famous Shiga toxin genes (*stx1* or *stx2*), or both of them. The enterohaemorrhagic *E. coli* (EHEC), which is a subset of STEC causes two prominent syndromes the hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Ferreira *et al.*, 2015; Castro *et al.*, 2017; Cundon *et al.*, 2018). The non-O157 STEC *E. coli* in small ruminants may lead to diarrhoea and gastrointestinal troubles while O157 can cause mucoid or bloody diarrhoea could progress to septicemia and meningoencephalitis. The characteristic post mortem findings are inflamed mucosa of the large intestine accompanied with a fibrinohemorrhagic exudate (ISUCFSPH, 2016). While signs in humans ranges from simple diarrhea to hemorrhagic colitis, there could be

progression to HUS, which is distinguished by the famous triad of thrombocytopenia, thrombotic microangiopathy, and hemolytic anemia in addition to grave acute renal failure (Mayer *et al.*, 2012; Momtaz *et al.*, 2013). The samples of ovine meat are highly contaminated with STEC types of *E.coli* O157 and non O157 and are sources of human infections (Momtaz *et al.*, 2013). The significant role of sheep and goats for spreading of STEC to human was suggested by many reports (Ferens and Hovde, 2011). The most notorious STEC serotypes encountered in human disease are *E. coli* O26, O45, O103, O111, O121, O145, and O157 (Scallan *et al.*, 2011). A huge arsenal of virulence factors are essential for the pathogenicity of STEC. From them the *stx1* toxin imparted similarity of the amino acid sequence with clear antigenic differences to *Shigella dysenteriae* serotype 1 cytotoxin (Beutin *et al.*, 1997; Djordjevic *et al.*, 2002). Based upon the previous epidemiological data the *stx2* exceeds *stx1* in its association with severe diseases and

HUS syndrome (Orth *et al.*, 2007; Kawano *et al.*, 2008). The Shiga toxin genes that encode for *stx1* and *stx2* which causes cell death after inhibiting the cellular protein synthesis. Recently, there are at least three *stx1* subtypes (*stx1a*, *c*, and *d*) and seven *stx2* subtypes (*stx2a*, *b*, *c*, *d*, *e*, *f*, and *g*) were recognized (Baylis, 2009).

The intimin encoded by *eaeA* gene, is an outer membrane essential for STEC attachment to intestinal cells. Furthermore, the enterohemolysins are encoded by *hlyA* or *ehxA* genes and that enhance the effect of Shiga toxins (Croxen *et al.*, 2013). In the last few years, there is an increased pathogenicity and resistance to most antimicrobials conferred by *E. coli* isolates that lower the selection chances of effective antimicrobial to control human infections (Saei *et al.*, 2012).

This research was aimed to isolate and identify STEC serotypes from adult and neonate sheep and goats either (healthy or showing diarrhea) and their bedding to carry out genotyping and detection of the vital virulence genes of the obtained isolates. In addition, the antimicrobial susceptibility testing was applied to screen for the resistance patterns of the obtained serotypes.

MATERIALS AND METHODS

Sampling: The collection of 170 samples was performed from sheep, goats, and bedding (136, 27 and 7) respectively. Samples were distributed as 136 fecal samples (34 diarrheic and 102 apparently healthy sheep and goats), 29 milk samples (25 sheep and 4 goats) and 7 bedding samples (5 sheep and 2 goats). The sample collection was carried out in a period from November 2015 to March 2016 from different sheep and goat farms at Badr City in Al-Buheria Governorate.

***E. coli* isolation:** The fecal samples were pre-enriched firstly in nutrient broth and kept for 24 hrs at 37°C, once growth confirmed a loopful of broth was streaked on MacConkey agar plates (MAC) and then aerobic incubation for 24 hrs at 37°C. The pink Lactose positive colonies were selected and cultivated on Eosin Methylene Blue (EMB) then incubated for 24 hrs at 37°C, typical *E. coli* colonies appeared metallic green. Positive colonies were stabbed into the semisolid medium and incubated for 24 hrs at 37°C, then, kept in the refrigerator at 4°C for preservation and for further morphological, biochemical, serological, and molecular characterization. The isolated *E. coli* strains upon specific media were determined at the species level using cytochrome oxidase, indole test, triple sugar iron agar, and urease (Quinn and Markey, 2003; Osman *et al.*, 2012). The *Staphylococcus aureus* ATCC 29737 and *E. coli* (O157:H7, *stx1*, *stx2*, *eaeA*, *hlyA*)

ATCC 35150 were utilized as negative and positive controls respectively.

Serological identification of *E. coli*: Antisera of *E. coli* specific to somatic "O" and flagellar "H" antigens utilizing the slide agglutination test were used for serological identification of isolates. The diagnostic antisera were supplied by Denka-Seiken (Japan). The conducted standard slide agglutination technique was performed according to the manufacturer's instructions at University of Banha, Faculty of Veterinary Medicine, The Center of Food Analysis.

Molecular characterization of virulence genes harbored by *E. coli* serotypes: The kept *E. coli* serotypes were cultivated on MAC agar plates, then overnight incubation at 37°C. After growth of culture, a sterile toothpick was used to pick up a single colony and the QIAamp kit was applied according to the manufacturer's instructions for DNA extraction. All the obtained *E. coli* serotypes were tested for the existence of virulence genes using primers targeting *Stx1*, *Stx2*, *eaeA*, and *hlyA* (Table 1).

Antimicrobial susceptibility patterns: Cultivation of serotypes was performed onto Muller-Hinton (Oxoid) broth for 18 hrs at 37°C, until the bacterial count was adjusted to 1x10⁶/1ml (using spectrophotometer). Once adjusted, then spreading on Mueller-Hinton agar (Oxoid) plates, then the disc diffusion method was performed using these antimicrobial disks: Amikacin (Ak 30), Amoxicillin /Clavulanic acid (AMC 30), Ampicillin (AM 10), Cephalexin (CTX 30), Cephadrine (CE 30), Chloramphenicol (C 30), Ciprofloxacin (CIP 5), Doxycycline (DO 30), Gentamycin (CN 10), Nalidixic acid (NA 30), Norocillin (Nor 10), Penicillin (P 10), Streptomycin (S 10), Trimethoprim/Sulphamethoxazole (SXT 25) (Oxoid). After incubation for 24 hrs at 37°C, the inhibition zones were measured and the results recorded as sensitive, intermediate, and resistant (Clinical Laboratory Standards Institute, 2013).

Statistical analysis: The STEC *E. coli* rates of isolation, the prevalence of the obtained serotypes and the sensitivity and resistance of isolates to antimicrobials were presented as percentages (%). The Multiple Antibiotic Resistance (MAR) index was displayed as a percentage of effective antimicrobials to the total used types. The significance of difference between STEC rates from sheep and goats, and between the serotypes exhibited MAR index ≤0.5 and MAR index >0.5 were 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: Oligonucleotide Primers used for amplification of specific genes

Primer	Oligonucleotide sequence (5'-3')	Product size (bp)	References	
<i>Stx1</i>	<i>stx1</i> (F)	ACACTGGATGATCTCAGTGG	614	(Fagan <i>et al.</i> , 1999; Osman <i>et al.</i> , 2012)
	<i>stx1</i> (R)	CTGAATCCCCCTCCATTATG		
<i>stx2</i>	<i>stx2</i> (F)	CCATGACAACGGACAGCAGTT	779	(Fagan <i>et al.</i> , 1999; Osman <i>et al.</i> , 2012)
	<i>stx2</i> (R)	CCTGTCAACTGAGCAGCATTG		
<i>eaeA</i>	<i>eaeA</i> (F)	GTGGCGAATACTGGCGAGACT	890	(Fagan <i>et al.</i> , 1999; Osman <i>et al.</i> , 2012)
	<i>eaeA</i> (R)	CCCCATTCTTTTCACCGTCCG		
<i>hlyA</i>	<i>hlyA</i> (F)	ACGATGTGGTTTATTCTGGA	165	(Fagan <i>et al.</i> , 1999; Osman <i>et al.</i> , 2012)
	<i>hlyA</i> (R)	CTTCACGTGACCATACATAT		

RESULTS

Prevalence of *E. coli* among examined samples: A total of 170 samples distributed as 141 from sheep comprising 111, 25, and 5 were fecal, milk and bedding samples, respectively. In addition to that, 29 goat samples comprising 23, 4, and 2 were fecal, milk and bedding samples respectively. All of them were subjected to isolation and biochemical identification using specific media. *E. coli* was detected at a rate of 71 (41.7%) which was distributed as 62 (44%) and 9 (31%) from sheep and goat samples, respectively, with no significant difference between these percentages ($P=0.1961$) (Table 2).

Detection of prevalent *E. coli* serotypes, virulence markers and antimicrobial susceptibility of serotyped isolates: From the obtained 71 *E. coli* isolates, 15 isolates were randomly selected for serotyping. The most prevalent serotypes were O111:H2, O26: H11, O103:H2, O55: H7, O86, O121:H7, O125:H21, and O124. The serotype O111:H2 (EHEC) was the most prevalent from one sheep fecal sample, 2 goat fecal samples and one sample from sheep bedding with a rate of 4 (26.7%). The O111:H2 harbored *stx1*, *stx2*, *eaeA*, and *hlyA* encoding virulence markers and exhibited Multiple Resistance Index (MAR) of 0.64 with resistance to AK30, AMC30, AM10, CIP5, CN10, CTX30, P10, SXT25, and S10. Furthermore, O26: H11 (EHEC) was isolated from 3 sheep fecal samples with a rate of 3 (20%) it contained *stx1*, *stx2*, *eaeA*, and *hlyA* virulence genes. The O26: H11 gave MAR of 0.21 with pronounced resistance to AM10, CTX30, and SXT25. Additionally, O103:H2 (EHEC) gained from one sheep fecal sample with a rate of 1 (6.7%) and marked by the existence of *stx1*, *stx2*, and *hlyA* genes. It gave MAR of 0.35 with clear resistance to AMC30, AM10, CN10, NA30, and P10. The serotype O55:H7 (EHEC) represented a rate of 2 (13.3%) one from the sheep fecal and one from sheep milk sample. It showed the highest MAR (0.71) of the isolated serotypes with resistance to AK30, AMC30,

AM10, CE30, CIP5, CN10, CTX30, DO30, S10, and P10. Both the O86 (EHEC) and O121: H7 serotypes were isolated with a similar rate of 1 (6.7%) from sheep fecal samples. Although, O86 contained *stx2* the serotype O121: H7 displayed *stx1* and *stx2* virulence genes. The serotype O86 gave MAR of 0.42 with resistance to CIP5, CN10, NA30, Nor10, S10, and SXT25. While, the serotype O121: H7 produced MAR of 0.50 with resistance to AK30, CIP5, CN10, CTX30, NA30, NOR10, and SXT25. The serotype O125: H21 (EHEC) was isolated with a rate of 2 (13.3%) representing 2 isolates from sheep fecal samples moreover; it owned *stx1* and *stx2* virulence genes. It gave MAR of 0.86 with resistance to AK30, AMC30, AM10, NA30, C30, CIP5, CN10, CTX30, SXT25, NOR10, P10, and S10. The last detected serotype was O124 that was isolated from one goat fecal sample with a rate of 1 (6.7%). It contained the *eaeA* gene and exhibited MAR of 0.86 with resistance to AK30, AMC30, AM10, CE30, CIP5, CN10, CTX30, DO30, NA30, P10, S10, and SXT25. It was lucid that all virulence genes were equally distributed in both the serotypes O111:H2 and O26:H11. Besides both O103:H2 and O55:H7 serotypes equally harbored the same virulence markers. The two serotypes O121:H7 and O125:H21 similarly gained two virulence markers, whereas O86 and O124 exhibited the lowest distribution pattern of virulence genes. The prevalence of *stx1* gene was 13/15 (86.7%), *stx2* was 14/15 (93.3%), the *eaeA* gene was 8/15 (53.3%), and *hlyA* gene was 10/15 (66.7%). The most effective antimicrobials were Chloramphenicol, Doxycycline and Cephadrine with sensitivity rates of 73.4%, 73.4%, and 73.3% respectively. Both the serotypes O125: H21 and O124 gave the highest MAR index while O55:H7, O111:H2, O121:H7, O86, and O103:H2 and O26:H11 showed MAR indices of 0.71, 0.64, 0.50, 0.42, 0.35, and 0.21 in a descending manner respectively. It was clear that 6/15 (40%) of the obtained serotypes exhibited MAR index ≤ 0.5 while 9/15 (60%) gave MAR index > 0.5 with a significant difference between them $P < 0.05$ using Fisher Exact Probability Test (Table 2 and 3).

Table 2: Prevalence of *E. coli* among examined samples

Species	No.	Breed	Management/efficiency	Types of samples				Isolated <i>E. coli</i> No. (%)
				Fecal		Milk	Bedding	
				Diarrheic	Healthy			
Sheep	141	local cross	Extensive/fattening	34(24.1%)	77(54.6%)	23(16.3%)	5 (3.6%)	62(44)
Goats	29	local cross	Extensive/ fattening	-	23(79%)	4(13.7%)	2(7.3%)	9(31)
Total	170			134		27	7	71(41.7)

Table 3: Antimicrobial susceptibility pattern of 15 *E. coli* serotypes isolated from examined sheep and goats

Antimicrobial agents	Resistance		Intermediate		Sensitive	
	No.	%	No.	%	No.	%
Amikacin (AK30)	9	60.0	4	26.7	2	13.3
Amoxicillin/Clavulanic acid (AMC 30)	7	46.6	0	0	8	53.4
Ampicillin (AM10)	10	66.7	0	0	5	33.3
Cefotaxime (CTX30)	13	86.7	2	13.3	0	0
Cephadrine (CE30)	4	26.7	0	0	11	73.3
Chloramphenicol (C30)	2	13.3	2	13.3	11	73.4
Ciprofloxacin (CIP5)	8	53.4	5	33.3	2	13.3
Doxycycline (DO30)	3	20.0	1	6.6	11	73.4
Gentamycin (CN10)	10	66.7	1	6.6	4	26.7
Nalidixic acid (NA30)	8	53.4	4	26.7	3	20.0
Norocillin (Nor10)	7	46.6	4	26.7	4	26.7
Penicillin (P10)	9	60.0	0	0	6	40.0
Streptomycin (S10)	8	53.4	2	13.3	5	33.3
Trimethoprim / Sulphamethoxazole (SXT25)	11	73.3	0	0	4	26.7

Table 4: Distribution of *E. coli* serotypes, virulence markers and antimicrobial susceptibility among obtained isolates from sheep and goats

Serotypes	Strain characterization	Type of sample	Sheep No(12)	Goat No(3)	Total No(15)	Virulence gene distribution patterns				Antimicrobial resistance patterns	Multiple Antibiotic Resistance (MAR) index
						<i>stx1</i>	<i>stx2</i>	<i>eaeA</i>	<i>hlyA</i>		
O111:H2	EHEC	Fecal sheep (1) Fecal goat (2) Bedding sheep (1)	2(16.7%)	2(66.7%)	4(26.7%)	+	+	+	+	AK30, AMC30, AM10, CIP5, CN10, CTX30, P10, SXT25, S10	0.64
O26:H11	EHEC	Fecal sheep (3)	3(25%)	—	3(20%)	+	+	+	+	AM10, CTX30, SXT25	0.21
O103:H2	EHEC	Fecal sheep (1)	1(8.3%)	—	1(6.7%)	+	+	-	+	AMC30, AM10, CN10, NA30, P10	0.35
O55:H7	EHEC	Fecal sheep (1) Milk sheep (1)	2(16.7%)	—	2(13.3%)	+	+	-	+	AK30, AMC30, AM10, CE30, CIP5, CN10, CTX30, DO30, S10, P10	0.71
O86	EHEC	Fecal sheep (1)	1(8.3%)	—	1(6.7%)	-	+	-	-	CIP5, CN10, NA30, Nor10, S10, SXT25	0.42
O121:H7	EHEC	Fecal sheep (1)	1(8.3%)	—	1(6.7%)	+	+	-	-	AK30, CIP5, CN10, CTX30, NA30, NOR10, SXT25	0.50
O125:H21	EHEC	Fecal sheep (2)	2(16.7%)	—	2(13.3%)	+	+	-	-	AK30, AMC30, AM10, NA30, C30, CIP5, CN10, CTX30, SXT25, NOR10, P10, S10	0.86
O124	EHEC	Fecal goat (1)	—	1(33.3%)	1(6.7%)	-	-	+	-	AK30, AMC30, AM10, CE30, CIP5, CN10, CTX30, DO30, NA30, P10, S10, SXT25	0.86

Amikacin (AK30), Amoxicillin/Clavulanic acid (AMC30), Ampicillin (AM10), Cephadrine (CE30), Ciprofloxacin (CIP5), Gentamycin (CN10), Cefotaxime (CTX30), Doxycycline (DO30), Nalidixic acid (NA30), Norocillin (NOR10), Penicillin (P10), Streptomycin (S10), Trimethoprim/Sulphamethoxazole (SXT25).

Ethical considerations: This study agrees with the U.S. Government rules for the employment and nursing of animals intended for experimental, training, and research objectives. The study design was accepted by the Faculty Committee for Animal Care and Use, University of Sadat City.

DISCUSSION

In Egypt, sheep and goat exhibit socioeconomic impact for meat, wool, and hide production, these species constituted about 5.6 and 4.13 million heads respectively. The wide field of investment and insurance in sheep and goat farming because of the adaptation to harsh conditions, high productivity, increased fertility, and short generation interval (Khalil *et al.*, 2013). *E. coli* is the most notorious bacterial agent causes drastic losses in sheep and goat farms. *E. coli* was detected at a rate of 71/170 (41.7%) which was distributed as 62/141 (44%) and 9/29 (31%) from sheep and goat samples, respectively, with no significant difference ($P=0.1961$). The highest trend of isolation was from sheep which comes in contradiction with Osman *et al.* (2013) who proved the vice versa. This high isolation rate was confirmed by Zaki *et al.* (2010) and Aklilu *et al.* (2013).

The STEC isolates were serotyped and the most prevalent serotypes were O111:H2, O26:H11, O103:H2, O55:H7, O86, O121:H7, O125:H21, and O124. These results were similar to that reported by Koch *et al.* (2001), Cookson *et al.* (2006), Mousa *et al.* (2010), and EL Malt *et al.* (2017). Most of these serotypes of public health classified as verotoxigenic serotypes associated with bloody diarrhea or hemolytic uremic syndrome in human (Johnson *et al.*, 1996; Delannoy *et al.*, 2012). The pathogenicity of STEC mainly ambuscades in a huge arsenal of virulence factors, including Shiga toxins (*stx1* and *stx2*, and its subtypes), adhesion protein intimin (*eae*), and enterohemolysin (*Ehly*) (Law, 2000; Delannoy *et al.*, 2012). The *eaeA* gene is present on chromosome at the

pathogenicity island; it is an essential factor for strong attachment to cells of the host intestinal mucosa. The attaching and effacing (A/E) lesions are a result of interaction between intimin and the bacterial “translocated intimin receptor” (Tir), this lesion is lacking in mutants that cannot colonize their hosts or cause lesions (Farfana and Torres, 2012). Furthermore, the enterohemorrhagic *E. coli* (EHEC) characterized by *hly* gene, that encodes for hemolysin which considered as a pore-forming cytolysin on eukaryotic cells (Islam *et al.*, 2008).

In this study, the distribution patterns of *stx1*, *stx2*, and *eaeA* were 13/15 (86.7%), 14/15 (93.3%) and 8/15 (53.3%) respectively, these results were higher than Cookson *et al.* (2006) who found that 43% of the tested cattle and sheep harbored *E. coli* isolates possessing *stx* and 33% *eaeA*. The frequency of the *hlyA* gene in the obtained STEC isolates in this study was 10/15 (66.7%) which confirmed by Lorenz *et al.* (2013) who found that most STEC strains (86.7%) were hemolytic. Commenting on the antimicrobial resistance pattern, it was noticeable that 6/15 (40%) of the obtained serotypes exhibited MAR index ≤ 0.5 while 9/15 (60%) gave MAR index > 0.5 with a significant difference between them $P < 0.05$. This result elucidated the high resistance pattern of the STEC isolates from sheep and goat with eminent focus on these isolates which represent a public health problem. This result confirmed by Mukherjee *et al.* (2017) who observed that there is a high frequency of antimicrobial drug resistance in O157 and non-O157 Shiga toxin-producing *E. coli*. Furthermore, the most effective antimicrobials were Chloramphenicol, Doxycycline, and Cephadrine with sensitivity rates of 73.4, 73.4 and 73.3%, respectively. Sheep and goat represent a possible reservoir of STEC serious to the public health due to the harbored virulence repertoire and the increased number of antimicrobial resistant *E. coli* isolates (Bai *et al.*, 2016). The monitoring of STEC antimicrobial resistance is pivotal due to the likelihood horizontal transmission of resistance genes from notorious STEC to other pathogens. The monitoring

process will aid in unraveling new treatment approaches and help in developing effective control strategies that help in stopping the spread of resistance (Islam *et al.*, 2008).

Conclusions: Our findings proved that sheep and goats call for special concern. Because both species represented a vital reservoir of Shiga-toxin producing *E. coli*, most of the obtained serotypes harbored many virulence genes and proved to be multiresistant to most used antimicrobials that considered crucial for public health. Hence, the molecular typing and contentious monitoring of antimicrobial resistance could be helpful for developing efficacious control strategies against STEC and for the formulation of new antimicrobials with lowered liability for antimicrobial resistance.

Authors contribution: MSAEE was the leader of this study, who planned, monitored and evaluated the research steps. He helped also in the sampling, the isolation, the genotyping, the antimicrobial susceptibility testing, the writing, the revision of the manuscript and the data analysis. AM helped in the sampling, the isolation, and most of the genotyping. AA and RT helped in the conceptualization of the study, gave some technical advice, and data analysis.

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