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The Distribution of *Escherichia coli* Serovars, Virulence Genes, Gene Association and Combinations and Virulence Genes Encoding Serotypes in Pathogenic *E. coli* Recovered from Diarrhoeic Calves, Sheep and Goat

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Summary

Ruminants, especially cattle, have been implicated as a principal reservoir of one of the enterovirulent *Escherichia coli* pathotypes. The detection of the virulence genes in diarrhoeic calves and small ruminants has not been studied in Egypt. To determine the occurrence, serotypes and the virulence gene markers, *stx1*, *stx2*, *hylA*, $Flic_{h7}$, *stb*, *F41*, *K99*, *sta*, *F17*, *LT-I*, *LT-II* and *eae*, rectal swabs were taken from diarrhoeic calves, sheep and goats and subjected to bacterial culture and PCR. The *E. coli* prevalence rate in the diarrhoeic animals was 63.6% in calves, 27.3% in goat and 9.1% in sheep. The 102 *E. coli* strains isolated from the calves, goat and sheep were 100% haemolytic non-verotoxic and fitted into the *Eagg* group. The isolates belonged to seven O serogroups (O25, O78, O86, O119, O158, O164 and O157). The *eae* gene was detected in six of the strains isolated from the calves. The 102 bovine, ovine and caprine *E. coli* strains isolated in this study were negative for *stx1*, *stx2*, *F41*, *LT-I* and $Flic_{h7}$ genes. The highest gene combinations were found to occur in the form of 24/102 isolates (23.5%) that carried the *F17* gene predominantly associated with *eaeA*, *hylA*, *K99* and *Stb* genes in the calves, while the *hylA*, *K99* and *Sta* were the only genes found to be in conjunction in both calves and goats (6/102; 5.9% each). Our data show that in Egypt, large and small ruminants could be a potential source of infection in humans.

Introduction

Escherichia coli is a member of the genus *Escherichia* within the family *Enterobacteriaceae*. Members of this family are widely distributed in the environment. *Escherichia coli* is usually a non-pathogenic member of the animal intestinal flora. As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals. References obtained from European Center for Disease Control and World Health Organization's epidemiological updates indicated that the health officials and World Health Organization (WHO) were worried enough about an unusually virulent outbreak of food-borne illness from the *E. coli* bacteria (Borgattaa et al., 2012). The epicentre of the outbreak was Northern Germany, from where it has spread throughout Germany and beyond, to other European countries (Frank et al., 2011; Rasko et al., 2011; Wieler et al., 2011; Wu et al., 2011; Beutin and Martin, 2012).

Strains of *E. coli* that cause enteric disease are termed enterovirulent (or diarrhoeagenic). However, certain strains have acquired virulence factors and may cause a variety of infections in humans and in animals (Stenutz et al., 2006).

Cattle and sheep represent a major reservoir for entry of pathogenic *E. coli* into the human population via the

food chain (Pradel et al., 2001). Ruminants, especially cattle, are the most frequent direct and indirect source of *E. coli* O157 (Bolton, 2011). Serotype O157 is responsible for approximately half of all confirmed diarrhoegenic *E. coli* infections in Europe (Ferens and Hoyde, 2011), but there is growing concern about the risk of non-O157 serotypes to human health. Diagnosing diarrhoegenic *E. coli* in humans, food and the environment has shown that non-O157:H7 serotypes are responsible for severe infections in humans (Blanco et al., 2004; Majalija et al., 2008). Non-O157 STEC strains are considered to be of greater clinical significance as causes of human disease than O157 strains in Uganda, Spain, Germany, France, Switzerland, Denmark, Belgium, Italy, Argentina, Australia, South Africa and Chile, but not in Canada, the United States, Japan, England or Scotland (Kehl, 2002; Park et al., 2002; Blanco et al., 2004).

Although the virulence genes of *E. coli* were recently investigated by Osman et al. (2012) in mastitic cattle, yet, to the authors' knowledge, the detection of the virulence genes in diarrhoeic calves and small ruminants has not been studied in Egypt. Therefore, the purpose of this study was to compare serotypes and virulence genes profiles from diarrhoeic calves, goats and sheep linked to human clinical cases (Badouei et al., 2010) by determining the presence or absence of the factors associated with virulence (*stx1*, *stx2*, *hylA*, $Flic_{h7}$, *stb*, *F41*, *K99*, *sta*, *F17*, *LT-I*, *LT-II* and *eae*).

Materials and Methods

Study design

The study was conducted from January to December during the year 2009. A total of 600 diarrhoeic calves ($n = 200$), goats ($n = 200$) and sheep ($n = 200$) from 35 farms on the outskirts of Cairo (geographical coordinate of 30°3′0″North, 31°15′0″East) were examined. The calves resided on 20 farms, 12 of which had one to four animals and eight of which had >50 animals. The small ruminants resided on 50 sheep and 16 goat farms. The mean herd size was 100 animals for sheep and 50 for goats. Both species are mainly reared under semi-extensive husbandry. The sheep farms produce milk, meat and wool, while the goat farms produce milk and meat. Most animals are born indoors and weaned when aged 1–3 months. Newborn animals go out to graze with their mother during the day. The farms had one or more animal (calf, goat or sheep) suffering from acute diarrhoea at the time of sampling. Sick calves, goat and sheep were selected randomly. The health status of each animal was evaluated by clinical examination. Sick calves, goat and sheep showed abnormal faecal consistency and/or signs of dehydration and weakness. None of the three species of animals had been vaccinated.

Sample collection

Faecal samples from 40 diarrhoeic calves (<3 months of age), 40 diarrhoeic goats (<1 month of age) and 20 diarrhoeic sheep (<2 months of age) were collected by placing approximately 10 g of faecal material obtained by rectal retrieval into Whirl-Pak bags. A separate glove was used for the collection of each sample. After collection, samples were shipped to a central laboratory at the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University. Samples were transported on the day of collection in styrofoam boxes with ice packs and were immediately processed.

Laboratory isolation and identification

Initial selection of *E. coli* isolates to be screened was conducted by adding 1 g of fresh faeces to 25 ml of enrichment medium [i.e. tryptic soy broth (TSB); Hardy Diagnostics, Santa Maria, CA, USA] containing 20 μg/ml novobiocin (Sigma, St. Louis, MO, USA) and 40 μg/ml vancomycin (Sigma). The faeces and medium were mixed vigorously, and the suspension was incubated at 25°C for 20 min to allow for antibiotic selection. Faecal suspensions were plated in duplicate onto sorbitol-MacConkey (SMAC) agar (Hardy Diagnostics) containing novobiocin (20 μg/ml) and vancomycin (40 μg/ml) and blood agar plates to assess the enterohaemolysin activity and incubated at 37°C for 18 h. *Escherichia coli* strains were identified using standard biochemical tests The presence of haemolytic or mucoid colonies was taken as evidence of *E. coli* pathogenicity. If there were multiple *E. coli* colonies available from a single sample, up to three different isolates were collected from the sample. Each isolate was suspended in tryptic soy broth (TSB), 0.5 ml of the suspension was added to 0.5 ml 65% glycerol solution, and the mixture was frozen at −70°C for further characterization studies.

Haemolytic activity

Escherichia coli produces two different haemolysins: the extracellular agent called α-haemolysin and a cell-bound β-haemolysin. The strains were tested for their haemolytic activity on agar base (Oxoid) supplemented with washed 5% sheep erythrocytes. The incubated plates were initially checked at 3 h. Haemolysis at this time on unwashed blood agar plates was regarded to be due to α-haemolysin, whereas haemolysis after overnight incubation indicated all types of haemolysis. Lysis on blood agar plates containing washed erythrocytes was regarded to be due to the presence of enterohaemolysin. This enterohaemolysin can only be observed on media prepared with washed sheep erythrocytes rather than whole sheep blood (Bettel-

heim, 1995). Five microlitres of each suspension was streaked onto the plates and incubated at 22 and 37°C for 24 h. The presence of a clear colourless zone surrounding the colonies indicated β-haemolytic activity (Vaishnavi et al., 2010).

Congo red dye uptake

The ability to take up Congo red dye was determined on agar plates supplemented with 50 mg/ml of Congo red dye. Five microlitres of each suspension was streaked onto the plates and incubated at 37°C for 24 h. Orange colonies were considered positive, and different intensities in the dye uptake were expressed as +, ++ and +++ (Paniagua et al., 1990).

O-serotyping

The somatic (O) antigen of *E. coli* strains was determined using O-antisera produced in rabbits by slide agglutination test. A collection of 11 O-serotypings were available at the Central Lab, Ministry of Health. This collection was established largely for the identification of strains of veterinary and human significance in Egypt and consisted of O25, O26, O78, O86, O111, O119, O126, O127, O157, O158 and O174.

Cytotoxicity assay

For production of Shiga toxins, one loopful of each isolated colony was inoculated in 50-ml Erlenmeyer flasks containing 5 ml of tryptone soy broth (pH 7.5) with mitomycin C (0.5 μg/ml), incubated for 20 h at 37°C (shaken at 200 rpm) and then centrifuged (6000 ***g***) for 30 min at 4°C. The Vero cell culture assay was performed using nearly confluent cell monolayers grown in plates with 24 wells. At the time of assay, the growth medium (RPMI with 50 units/ml of polymyxin sulphate) was changed (0.5 ml per well), and 75 μl of undiluted culture supernatant added. Cells were incubated at 37°C in a 5% CO_2 atmosphere, and the morphological changes in cells observed after 24 and 48 h of incubation using a phase contrast inverted microscope (Blanco et al., 2004). The highest toxin dilution that caused lysis of 50% of the cell monolayer was taken as the titre.

Detection of virulence determinants (stx1, stx2, hylA, $Flic_{h7}$, stb, F41, K99, sta, F17, LT-I, LT-II and eae) by PCR

DNA isolation

Escherichia coli strains were subcultured onto MacConkey agar plates, utilizing quadrant streaking methods to produce isolated colonies. These strains were incubated at 37°C for overnight. A single colony was carefully removed from the plate by using a sterile toothpick to avoid agar contamination, an important cause of erratic amplification. Crude lysates were prepared and used directly as a template for the PCR. DNA was extracted by boiling a single colony in 50 μl of PCR molecular-grade water for 5 min, followed by centrifugation at 20 800 ***g*** for 10 min. Two microlitres of this lysate was used as a template with a 23 μl of PCR master mix to make a 25-μl total reaction volume.

Reference *E. coli* strain ATCC35150 (O157:H7, *stx*1, *stx*2, *eae*, *hly*) and *E. coli* HB 101 inv^+ were used as a positive control, and *S. aureus* ATCC29737 as a negative control (Chart et al., 2000).

Polymerase chain reaction

The *stx*1, *stx*2, *hylA*, $Flic_{h7}$, *stb*, *F41*, *K99*, *sta*, *F17*, *LT-I*, *LT-II* and *eaeA* genes were detected using the primers and PCR conditions described in Table 1. All oligonucleotides were diluted to 20 pmol/ml and stored at −22°C. Bacterial DNA amplification was performed in 50 ml (total volume) of sterile, distilled water containing 2 ml of bacterial lysates (100 mg DNA/ml), 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM of $MgCl_2$, 40 pmol of each oligonucleotide primer, 0.5 ml of 103 PCR buffer and 1.25 U of *Taq* polymerase. The *E. coli* strains were subjected to s-PCR, performed in an Eppendorf Mastercycler (Ependorf AG, Hamburg, Germany). Control DNA samples from reference strains were included in each reaction. The PCR product was observed after electrophoresis on 1.2% agarose gel and staining with ethidium bromide. The results were recorded using an Alpha Imager documentation camera.

Results

A total of 132 *E. coli* isolates were recovered from faeces of diarrhoeic animals (calves $n = 84$, goats $n = 36$ and sheep $n = 12$ isolates in a prevalence rate of 63.6%, 27.3% and 9.1%, respectively) collected from different farms in Lower Egypt (Delta) during 2009.

The *E. coli* isolates were analysed for their somatic antigen (serogroup) where 77.3% (102/132 isolates) belonged to seven O serogroups (O25, O78, O86, O119, O158, O164 and O157) and 22.7% (30/132 isolates) belonged to a non-identified serogroup.

Expression of STx was examined by Vero cell cytotoxic assay. None of the 132 strains examined were able to exhibit cytotoxicity on the Vero cells, although they were 100% haemolytic.

All of the 132 isolates showed a haemolytic phenotype that resembled α-haemolytic activity rather than the

Table 1. Virulence factor targets and primers, including nucleotide sequences, PCR conditions and references

Target	Specificity	Nucleotide sequence of primers	PCR conditions[a]			Amplicon (bp)	References
			Denaturing	Annealing	Extension		
Eagg	Enteroaggregative	5′-AGACTCTGGCGAAAGACTGTATC-3′	95°C for 30 s	63°C for 30 s	72°C for 30 s[b]	194	Pass et al. (2000)
Eagg		5′-ATGGCTGTCTGTAATAGATGAGAAC-3′					
Einv	Enteroinvasive	5′-TGGAAAAACTCAGTGCCTCTGCGG-3′				140	
Einv		5′-TTCTGATGCCTGATGGACCAGGAG-3′					
Stx1	Shiga toxin 1	5′- ACA CTG GAT GAT CTC AGT GG-3′	94°C 60 s	58°C 45 s	72°C 90 s[c]	614	Sheng et al. (2005)
Stx1		5′- CTG AAT CCC CCT CCA TTA TG-3′					
Stx2	Shiga toxin 2	5′- CCA TGA CAA CGG ACA GCA GTT-3′		60°C 45 s		779	
Stx2		5′- CCT GTC AAC TGA GCA GCA CTT TG-3′					
hylA	Haemolysin	5′- ACG ATG TGG TTT ATT CTG GA-3′		52°C 45 s		165	
hylA		5′- CTT CAC GTG ACC ATA CAT AT-3′					
$Flic_{h7}$	H7 flagellar antigen	5′- GCG CTG TCG AGT TCT ATC GAGC-3′		60°C 45 s		625	
$Flic_{h7}$		5′- CAACGGTGACTTTATCGCCATTCC-3′					
Stb	Heat-stable	5′- GCG TCC CTG CGT ATC AGT AT-3′		57°C 45 s		241	Guler and Gunduz (2007)
Stb	enterotoxin b (Stb)	5′- CTT TTA AGG CAA GCG TCG TC-3′					
F41	Fimbrial F antigens	5′-GCA TCA GCG GCA GTA TCT-3′	94°C 30 s	53°C	70°C 90 s[d]	380	
F41		5′- GTC CCT AGC TCA GTA TTA TCA CCT-3′					
K99	K99	5′- TAT TAT CTT AGG TGG TAT GG-3′		53°C		314	
K99		5′- GGT ATC CTT TAG CAG CAG TAT TTC-3′					
Sta	Heat-stable	5′- GCT AAT GTT GGC AAT TTT TAT	94°C 60 s	53°C	72°C 90 s[c]	190	
	enterotoxin a (Sta)	TTC TGT A-3′					
Sta		5′- AGG ATT ACA ACA AAG TTC ACA GCA GTA A-3′					
P7	F17 fimbrial genes.	5′-CGG AGC TAA TAC TGC ATC AAC C-3′	94°C 120 s	55°C 60 s	70°C 60 s[d]	615	
P8		5′-CGT GGG AAA TTA TCT ATC AAC G-3′					
P9		5′-TGT TGA TAT TCC GTT AAC CGT AC-3′					
LT-I	Enterotoxins LT-I	5′- TAT CCT CTC TAT ATG CAC AG-3′	94°C 30 s	48°C 45 s	70°C 90 s[d]	480	Salvadori et al. (2003)
LT-I		5′- CTG TAG TGG AAG CTG TTA TA-3′					
LT-II	Enterotoxins LT-II	5′- AGA TAT AAT GAT GGA TAT GTA TC-3′	94°C 30 s	48°C 45 s		300	
LT-II		5′-TAA CCC TCG AAA TAA ATC TC -3′					
eae	Intimin	5′- GACCCGGCAACAAGCATAAGC-3′	94°C 30 s	55°C 45 s	70°C 90 s[d]	384	
eae		5′- CCA CCT GCA GCA ACA AGA GG-3′					

[a]Unless stated otherwise, PCR was carried out for 25 cycles.
[b]After 20 cycles, final extension step of 5 min at 72°C was performed.
[c]After 35 cycles, final extension step of 10 min at 72°C was performed.
[d]After 25 cycles, final extension step of 10 min at 70°C was performed.

typical enterohaemolytic phenotype. The zone was clear and large and could be visualized after 3–18 h incubation at 37°C. Of the 102 strains, 84 were positive by PCR using E-*hlyA* primer pair.

Congo red (CR) assay was used as a phenotypic marker for the invasive and non-invasive *E. coli*. In this study, all of the 132 tested serovars for the CR binding affinities were 100% positive. The binding activity of the CR dye was found to be variable in their affinity according to their serovars.

The results of the PCR (Fig. 1a) revealed a negative result for the amplification of the 140-bp fragment for the *Einv* gene in the 102/132-serotyped *E. coli* field isolates in an incidence of 100%. On the other hand, the 102/132-serotyped *E. coli* field isolates revealed positive amplification for the 194-bp fragment of the *Eagg* gene from the extracted DNA of the *E. coli* isolates in an incidence of 100%.

The 102/132-serotyped *E. coli* strains were submitted to PCR to detect *stx1*, *stx2*, *hylA*, $Flic_{h7}$, *eae*, *F41*, *K99*, *Sta*, *Stb*, *F17*, *LT-I* and *LT-II* genes, and the results are recorded in Table 2 and Fig. 1b–h.

The most prevalent distribution pattern of the detected virulence genes in the calves, sheep and goat was observed to be *hylA* (66/102; 64.7%), *K99* (54/102; 52.9%), *F17* (48/102; 47.1%), *Stb* (42/102; 41.2%) in the calves. On the other hand, prevalence of *Sta* (18/102; 17.6%) was similar in the calves and goat. With the same percentage of 17.6 (18/102), *hylA* was detected in the goat.

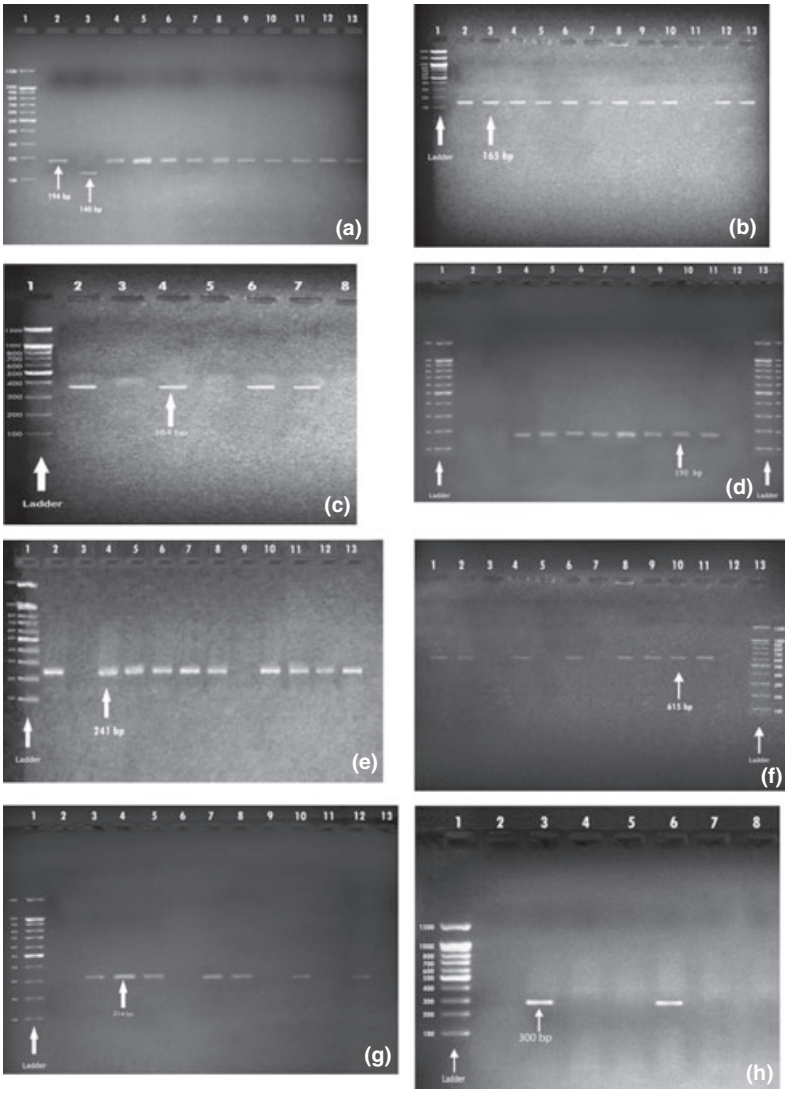

Fig. 1. Agarose gel electrophoresis showing amplification of the: (a) 194-bp and the 140-bp fragments for the *Eagg* and *Einv* genes of *Escherichia coli* performed with their specific primers. Lane 1: 100-bp molecular weight ladder. Lane 2: control *E. coli* HB 101 inv$^+$. Lane 3: Positive control (*E. coli* ATCC35150 serotype O157:H7). Lanes 4 and 5: Positive amplification of the 194-bp fragment. All of the bacterial strains showed positive amplification for the *Eagg* gene of *E. coli*. (b) 165-bp fragments using PCR with a primer specific for the *hylA* gene of *E. coli*. Lane 1: 100 bp molecular weight ladder. Lane 11: Negative control (*S. aureus* ATCC 29737). Lanes 2–10 and 13: Positive amplification of 165-bp fragments of *E. coli* field isolates. Lane 12: Positive control (*E. coli* ATCC35150 serotype O157:H7). (c) 384-bp fragments using PCR with a primer specific for the intimin gene (*eaeA*) of *E. coli*. Lane 1: 100-bp molecular weight ladder. Lane 2: Positive control (*E. coli* ATCC35150 serotype O157:H7). Lane 3: Negative control (*S. aureus* ATCC 29737). Lanes 4, 6, 7: Positive amplification of 384-bp fragments of *E. coli* field isolates. (d) 190-bp fragments using PCR performed with a primer specific for the *sta* gene of *E. coli*. Lanes 1 and 13 show 100-bp molecular weight ladders. Lane 2: Negative control (*S. aureus* ATCC 29737). Lanes 4, 5, 6, 7, 8, 9, 10, 11 show positive amplification of 190-bp fragments of *E. coli* field isolates. (e) 241-bp fragments using PCR with a primer specific for the *stb* gene of *E. coli*. Lane 1: 100-bp molecular weight ladder. Lane 2: Positive control (*E. coli* ATCC35150 serotype O157:H7). Lane 3: Negative control (*S. aureus* ATCC 29737). Lanes 4, 5, 6, 7, 8, 10, 11, 12 and 13: Positive amplification of 241-bp fragments of *E. coli* field isolates. (f) 615-bp fragments using PCR performed with a primer specific for the *F17* gene of *E. coli*. Lane 13: 100-bp molecular weight ladder. Lane 3: Negative control (*S. aureus* ATCC 29737). Lanes 1, 2, 4, 6, 8, 9 and 10: Positive amplification of 615-bp fragments of *E. coli* field isolates. Lane 11: Positive control (*E. coli* ATCC35150 serotype O157:H7). (g) 314-bp fragments using PCR performed with a primer specific for the *k99* gene of *E. coli*. Lane 1: 100-bp molecular weight ladder. Lane 2: Negative control (*S. aureus* ATCC 29737). Lane 3: Positive control (*E. coli* ATCC35150 serotype O157:H7). Lanes 4, 5, 7, 8, 10, 12: Positive amplification of 314-bp fragments of *E. coli* field isolates. (H) 300-bp fragments of the *LT-II* gene performed with primers specific for the *LT-I* and *LT-II* genes of *E. coli*. Lane 1: 100-bp molecular weight ladder. Lane 2: Negative control (*S. aureus* ATCC 29737). Lanes 3 and 6: Positive amplification of 300-bp fragments of *E. coli* field isolates. All *E. coli* field isolates were negative for *LT-I* gene.

Table 2. The distribution of *Escherichia coli* serovars, virulence genes, gene association and combinations and virulence genes encoding serotypes in pathogenic *E. coli* recovered from diarrhoeic calves, sheep and goat

	Animals		
	Calves (%)	Sheep (%)	Goat (%)
Distribution of E. coli *serovars*			
O25:K-	6/102 (5.9)	0	0
O78:K80	6/102 (5.9)	0	6/102 (5.9)
O86:K61	24/102 (23.5)	0	6/102 (5.9)
O119:K69	6/102 (5.9)	0	0
O157:K-	18/102 (17.7)	0	0
O158:K-	6/102 (5.9)	6/102 (5.9)	12/102 (11.8)
O164:K-	0	0	6/102 (5.9)
Un-typable	18/102 (17.7)	6/102 (5.9)	6/102 (5.9)
Distribution pattern of the detected virulence genes			
hylA	66/102 (64.7)	0	18/102 (17.6)
eaeA	6/102 (5.9)	0	0
Sta	18/102 (17.6)	0	18/102 (17.6)
Stb	42/102 (41.2)	6/102 (5.9)	12/102 (11.8)
F17	48/102 (47.1)	0	6/102 (5.9)
K99	54/102 (52.9)	6/102 (5.9)	12/102 (11.8)
LT-II	0	6/102 (5.9)	6/102 (5.9)
Gene association and combinations			
hylA/Stb	12/102 (11.8)	0	0
Sta/K99	0	0	6/102 (5.9)
Sta/F17	0	0	6/102 (5.9)
hylA/Sta/K99	6/102 (5.9)	0	6/102 (5.9)
hylA/Stb/K99	0	0	6/102 (5.9)
hylA/Stb/F17/K99	6/102 (5.9)	0	0
Stb/K99/LT-II	0	6/102 (5.9)	0
hylA/Stb/K99/LT-II	0	0	6/102 (5.9)
eaeA/hylA/Stb/F17/K99	24/102 (23.5)	0	0
hylA/Sta/F17/K99	18/102 (17.6)	0	0
Virulence genes and encoding serotypes in pathogenic E. coli			
Serogroups/genes			
O25:K-/*hylA*	6/102 (5.9)	0	0
O25:K-/*Stb*	6/102 (5.9)	0	0
O25:K-/*K99*	6/102 (5.9)	0	0
O25:K-/*F17*	6/102 (5.9)	0	0
O78:K80/*hylA*	6/102 (5.9)	0	6/102 (5.9)
O78:K80/*Stb*	6/102 (5.9)	0	6/102 (5.9)
O78:K80//*K99*	0	0	6/102 (5.9)
O86:K61/eaeA	6/102 (5.9)	0	0
O86:K61/*hylA*	24/102 (23.5)	0	0
O86:K61/*Sta*	0	0	6/102 (5.9)
O86:K61/*Stb*	24/102 (23.5)	0	0
O86:K61/*K99*	24/102 (23.5)	0	6/102 (5.9)
O86:K61/*F17*	24/102 (23.5)	0	0
O157:K-/*hylA*	18/102 (17.6)	0	0
O157:K-/*Sta*	18/102 (17.6)	0	0
O157:K-/*K99*	18/102 (17.6)	0	0
O157:K-/*F17*	18/102 (17.6)	0	0
O158/K-/*K99*	0	0	6/102 (5.9)
O158:K-/*hylA*	6/102 (5.9)	0	6/102 (5.9)
O158:K-/*Sta*	6/102 (5.9)	0	12/102 (11.8)
O158:K-/*Stb*	0	6/102 (5.9)	0
O164:K-/*hylA*	0	0	6/102 (5.9)
O164:K-/Stb	0	0	6/102 (5.9)
O164:K-/*K99*	0	0	6/102 (5.9)
O164:K-/LT-II	0	0	6/102 (5.9)

The rest of the assayed genes, *stx1*, *stx2*, *F41*, *LT-I* and $Flic_{h7}$, were not detected in any of the animals.

The highest gene combinations were found to occur in the form of 24/102 isolates (23.5%) that carried the *F17* gene predominantly associated with *eaeA*, *hylA*, *K99* and *Stb* genes in the calves. While the *hylA*, *K99* and *Sta* were the only genes found to be in conjunction in both calves and goats (6/102; 5.9% each), in the sheep, the genes were revealed as one combination.

Six isolates in each of the calves and goats contained the *hylA* gene encoding serotype O78:K80 and O158:K-. The same rate was found in both animals with the O78:K80/*Stb*. Both animals were again found to carry the same combinations where *K99* encoded serogroup O86:K61 (24/102; 23.5% and 6/102; 5.9% in calves and goats, respectively). *Sta* encoded serogroup O158:K- (6/102; 5.9% and 12/102; 11.8% in calves and goats, respectively). The only typable serotype in the sheep, O158:K-, was encoded by the gene *Stb* absent in the calves and goats.

Discussion

The prevalence rate of pathogenic *E. coli* in our study was much lower (63.6% in calves, 27.3% in goat and 9.1% in sheep) than previous studies (Cid et al., 1996; Novotna et al., 2005; Cookson et al., 2006).

Ruminants, especially cattle, constitute a vast reservoir of enterovirulent *E. coli*. The practice of applying either slurry or fresh manure to land has been shown to transfer pathogenic *E. coli* to food crops and surface water, consequently causing human infection (Meng and Schroeder, 2007). Although O157 was found to be, at least occasionally in a transient and seasonal manner, present on most farms (75–90%) of America and Europe (Blanco et al., 2001; Hussein et al., 2003), yet we recorded it in one sample only.

Cases of natural and experimental enteric colibacillosis have been reported infrequently in small ruminants (Bolton et al., 2009; Kiranmayi et al., 2010). Worldwide, goat and sheep have been shown to shed several diverse non-O157 serotypes in their faeces. Several of these serotypes have been associated with sporadic cases or major

outbreaks of human illnesses (Stockbine et al., 1998). The recorded serogroups identified in our study are of a different diversity than previous records (Bettelheim et al., 2000; Bonardi et al., 2004; Vu-Khac and Cornick, 2008), but represent serotypes that are commonly recovered from humans with serious disease in our community.

Testing by Vero cell cytotoxicity assay, considered to be the 'gold standard' for the detection of STx (Konowalchuk et al., 1977), was adopted in our investigation, as the ELISA was found to reflect a lower sensitivity compared with the Vero cell assay (Brooks et al., 2001). Beutin et al. (1989) observed a close association between cytotoxicity and production of haemolysin (Enterohaemolysin; *Ehly*). Bettelheim (1998, 2001) realized that non-O157 VTEC are as important as O157 VTEC. However, it should be noted that not all *Ehly*-positive strains are VTEC. Eventually, although the isolates in the present study were 100% haemolytic, yet no cytopathic effect was observed with the *E. coli* field isolates tested.

All diarrheagenic *E. coli* carry at least one virulence-related property. The virulence mechanisms that characterize *E. coli* are genetically coded for by chromosomal, plasmid and bacteriophage DNAs and include heat-labile (*LTI*, *LTIIa* and *LTIIb*) and heat-stable (*STI* and *STII*) toxins, verotoxin types 1, 2 and 2e (*VT1*, *VT2* and *VT2e*, respectively), attaching and effacing mechanisms (*eaeA*), enteroaggregative mechanisms (*Eagg*) and enteroinvasive mechanisms (*Einv*) (Pass et al., 2000).

By the aid of the PCR, the *E. coli* isolates from the calves, goat and sheep described herein fitted into the *Eagg* group. The *Eagg* classification of this isolate is further supported by its inability to demonstrate any cytopathic activity on cultured Vero cells.

In the present study, *eae* was detected in one faecal sample. The low prevalence of *eae* gene has been reported in many studies (Hornitzky et al., 2005; Fremaux et al., 2006). The importance of this data lies in the fact that *eae*-positive strains are considered more virulent for humans than *eae*-negative strains. However, most pathogenic *E. coli* strains isolated from the faeces of cattle, goat and sheep do not possess the *eae* gene (Kobayashi et al., 2001; Pradel et al., 2001; Blanco et al., 2004; Vu-Khac and Cornick, 2008). Several authors (Hall et al., 1990; Brett et al., 2003; Mercado et al., 2004; Hornitzky et al., 2005) found that the classic *stx1*, *stx2* and *eae* were carried on the O5 from diagnostic bovine faecal samples. However, it must be noted that O5 was not recovered in our study, and instead, the *eae*A gene was carried on the O86 isolated from the diarrhoeic calf.

Globally, faecal samples from diarrhoeic calves carried the genes *stx1*, *stx2*, *eaeA*, *Ehly* and combinations of stx1 and *stx2*, *stx1* and *eae*, and *stx*2 and *eaeA* genes in their faeces (Herrera-Luna et al., 2009; Alexa et al., 2011; Nguyen et al., 2011). In our study, the *stx*1 and *stx*2 genes were undetected, in contrast to all previous reports, while the *hly*A gene prevailed. Schmidt et al. (1995) reported the genetic analysis of a new plasmid-encoded haemolysin, *Ehly*, associated with severe clinical diseases in humans (Schmidt et al., 1996). Beutin et al. (1989) studied haemolysin production in a large number of serologically diverse VT+ *E. coli* strains and found an association between enterohaemolysin and verotoxin production in 89% of *E. coli* strains belonging to nine different serotypes. A suggestion was raised that enterohaemolysins may complement the effects of shiga toxins enhancing their virulence (Nataro and Kaper, 1998).

On the other hand, our results were consistent with the results of Dunn et al. (2003) where their isolates were PCR-positive for *fliC*H7, *eae*A and *hly*A, and Nguyen et al. (2011) indicated that calves were found to possess at least one of the fimbrial antigens.

Novel combinations of virulence factors may be rapidly detected in established pathogenic serovars of *E. coli* isolated from symptomatic animals. Accordingly, different gene combinations were encountered in our investigation as recorded in Table 2. *Escherichia coli* with at least one virulence factor and different expression frequencies was frequently isolated from diarrhoeic calves, goat kids and lambs (Fremaux et al., 2006; Warish et al., 2007; Vu-Khac and Cornick, 2008; Ok et al., 2009).

Most enterotoxigenic *E. coli* strains isolated from ruminants are *F41*+ or *F5*+ and produce *ST*I (Woodward and Wray, 1990). The reason for this association is that both virulence factors are generally encoded in the same plasmid. The absence of *LT*-I in the strains isolated in the present investigation is not surprising as *LT*-I is considered atypical in ruminant strains (Woodward and Wray, 1990).

Collectively, our data indicate that diagnostic bovine faeces are a source of pathogenic *E. coli* with serotypes and virulence genes that show many similarities to those identified from human patients with a range of gastrointestinal and other serious diseases. However, we isolated only one strain, O86, carrying the *eae* gene, contrary to previous studies, recovered from diarrheagenic calves, which indicated that a significant number possess *eae* (Kobayashi et al., 2003). Although most *eae*-positive strains isolated from diarrheagenic cattle in different regions of the world possess *stx*1 (Kobayashi et al., 2003; Hornitzky et al., 2005; Majalija et al., 2008), yet we found that the one *eae*-positive isolate in our samples did not possess *stx*1 or/and *stx*2 and does not keep with these observations. A role for *hylA*-positive *E. coli* as the cause of diarrhoea and other gastrointestinal afflictions in humans is gaining momentum (Trabulsi et al., 2002), and it is clear that diarrheagenic calves, goat and sheep could pose an important source of these potential pathogens for the time being in Egypt.

In conclusion, our data show that, in Egypt, cattle, goats and sheep could be a potential source of infection to humans, as the important serotypes previously described and associated with severe diseases in humans, such as O26, O86, O111, O126, O127, O157, O158 and O164, were isolated in addition to the virulence markers carrying at least one virulence-related property affecting the virulence of diarrheagenic *E. coli* in Egypt. Thus, in addition to the calves, goats and sheep may have a zoonotic potential and may serve as a source of *Eagg* for transmission to humans via contaminated meat and milk. Therefore, efforts should be focused on infection control to reduce the prevalence of such microorganisms. Direct contact with cattle, goats and sheep or consumption of water or foodstuffs contaminated with cattle, goat or sheep faeces may represent a significant source of infection for humans. Therefore, adequate manure management is one of the methods that prevent bovine, caprine or ovine faeces from becoming a source of environmental contamination with shigatoxigenic *E. coli*. Stress prevention, controlling food and water quality, as well as feedlot conditions and contact between adult and young animals should be taken into consideration in the control measures.

Conflicts of interest

None of the authors (Kamelia M Osman, Ashgan M Mustafa, Mahmoud Elhariri, Ghada S AbdElhamed) has a financial or personal relationship with other people or organisations that could inappropriately influence or bias our paper entitled 'The distribution of *Escherichia coli* serovars, virulence genes, gene association and combinations and virulence genes encoding serotypes in pathogenic *E. coli* recovered from diarrhoeic calves, sheep and goat'.

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