Original Article

Genotypic characterization of Egypt enterotoxigenic *Escherichia coli* isolates expressing coli surface antigen 6

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Abstract

Introduction: One approach to control enterotoxigenic *Escherichia coli* (ETEC) infections has been to develop vaccines focused on inducing protective immunity against surface expressed antigenic factors. One such factor is coli surface antigen 6 (CS6); ETEC isolates expressing CS6 may also simultaneously co-express surface antigens CS4 or CS5. However, there is little information regarding the inter-relationships of isolates expressing the CS6 antigen alone or in combination with CS4 or CS5.

Methodology: A total of 62 CS6-associated ETEC isolates were evaluated for their antimicrobial susceptibility, mechanisms of resistance, toxin genes, colonization factor expression, and XbaI-pulsed-field gel electrophoretic profiles.

Results: We observed 46 XbaI profiles; 31 were exclusive to ETEC expressing CS6 alone and 15 among the ETEC co-expressing CS4 or CS5. Nearly half (47%) of these isolates were resistant to ampicillin, a third (37%) of the isolates were resistant to trimethoprim-sulfamethoxazole, and 24% of the isolates were tetracycline-resistant. A bla_{TEM} gene was detected in 24 (83%) ampicillin-resistant isolates. Trimethoprim-sulfamethoxazole-resistant isolates (n = 23) carried either *sulI* (n = 1, 4%), *sulII* (n = 8, 35%) or both genes (n = 10, 43%); 4 had no detectable *sul* gene.

Conclusion: Our results show a lack of clonality among Egypt CS6 *E. coli* isolates and supports the use and the further research on vaccines targeting this cell surface antigen.

Key words: Enterotoxigenic Escherichia coli; children; diarrhea; Egypt

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Introduction

Enterotoxigenic *Escherichia coli* [1] is a significant global cause of diarrhoea in children living in developing countries [2] as well as travellers [3]. Diarrhoea results from the secretion of ETEC heat-labile (LT) and/or heat-stable (ST) enterotoxins [4]. Prior to toxin secretion, ETEC organisms must colonize the mucosal epithelium; this process utilizes fimbrial and non-fimbrial colonization factors, also referred to as coli surface antigens (CS) [4]. Over 21 ETEC CS have been described [5]. Individual CS expression may occur alone as is the case with CFA/I, or more than one CS may be co-expressed, such as CS6 together with the CS4 or CS5 fimbriae [6].

CS6 is one of the most prevalent ETEC CS observed worldwide [7-10] and has received considerable attention as a target for vaccine development [11-14]. CS6 is immunogenic in humans both after natural infection and after vaccination with an oral inactivated ETEC vaccine containing formalin-inactivated CS6-expressing bacteria, giving rise to IgM, IgG and IgA responses [15,16]. CS6 may be expressed alone or in combination with two other CS, CS4 and CS5. A previous study in Egypt showed that 79/915 (8.6%) ETEC isolates recovered from children expressed CS6 alone, whereas15 isolates (1.6%) expressed CS4/CS6 and 14 isolates (1.5%) expressed CS5/CS6 [8]. Qadri *et al.* recovered a greater percentage of CS6-expressing isolates (n = 114) from 381 ETEC recovered from children in Bangladesh [10]. Nearly equal proportions of CS4/CS6 (n = 40; 10%), CS5/CS6 (n = 39; 10%), and CS6 alone (n = 35;

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9.2%) expressing isolates were recovered. In Argentina, Binzstein *et al*. (1991) recovered 19 CS6-expressing isolates from 109 ETEC recovered from children aged 4 years and under [9]. The majority of CS6 isolates co-expressed CS5 (n = 16); very few expressed CS6 alone (n = 3) and none co-expressed CS4 [9]. The reason for variability in recovery of CS6 co-expressing isolates is not obvious, but it may be due to different selective pressures and detection methods used in the studies.

To understand the epidemiology and spread of ETEC in an endemic environment it is necessary to characterize the inter-relationship of the isolates. This information can be used to understand the organism's ecology and subsequently control the spread of disease. It may also be possible to use this information to identify regionally important strains and track their dissemination on a global level. Expressed traits such as lipopolysaccharide (O), flagellar (H), and capsular (K) antigens are commonly used to group ETEC into distinct serotypes; toxin- and CS-expression have been used to further differentiate these isolates [8,9,17,18].

Antimicrobial agents such as sulfonamides and beta-lactam-containing compounds can be an effective prophylaxis against traveller's diarrhoea caused by ETEC and in the absence of effective vaccines, treatment with antimicrobials remains the standard of care for many travellers [21]. However, concerns over the emergence of antimicrobial resistant strains due to patient self-treatment and abuse of the therapeutic agents discourage this practice [19,20]. Genes encoding resistance proteins to commonly used antimicrobial agents in the developing world, such as sulfonamides and beta lactam antimicrobials, are known to reside on horizontally transmissible elements such as conjugative plasmids and integrons, promoting the spread of resistance [60,61].

The present study investigated the diversity of Egypt CS6-expressing ETEC by comparing XbaI-pulsetypes and the mechanisms of resistance to selected antimicrobial agents in a collection of previously serotyped ETEC CS6-expressing isolates obtained from children younger than 3 years of age participating in a longitudinal, community-based study [8,22].

Methodology

Bacterial strains and culture conditions

During a prospective study conducted from 1995-1998, rectal swab specimens were obtained from a cohort of children with diarrhoea residing in the village of Abu Homos, El-Behira Governorate, Egypt. The Abu Homos field site has been described previously [22]. Five individual lactose fermenting colonies with typical morphology of *E. coli* were isolated from MacConkey-lactose agar medium, cultured overnight in brain heart infusion broth [23], and frozen in 15% glycerol at -70ºC until use. *E. coli* and other enteric bacterial pathogens (*i.e.*, *Campylobacter*, *Salmonella* spp., *Shigella* spp.) were identified by standard procedures and API20E [24]. Expression of ETEC CS was detected using a panel of monoclonal antibodies as previously described [8,9].

Phenotype characteristics of antimicrobial resistance

CS6-expressing ETEC isolates were characterized for their antimicrobial resistance phenotypes to 14 commonly used antimicrobials by the disk diffusion method of Kirby-Bauer [25] on Mueller-Hinton agar (Becton Dickinson, Cockeysville, MD, USA). Zone diameters obtained for each isolate to each antimicrobial agent tested were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute [26]. The antimicrobial susceptibility testing panel included ampicillin (AMP) 10 µg, AMP 10 ug / sulbactam (SAM) 10 µg, ticarcillin 75µg / clavulanic acid (TIM) 10 µg, cefepime (FEB) 30µg, ceftriaxone (CRO) 30 µg, ceftazidime (CAZ) 30µg, nalidixic acid (NAL) 30 µg, ciprofloxacin (CIP) 5 µg, amikacin (AN) 30 µg, gentamicin (GEN) 10 µg, tetracycline (TET) 30 µg, trimethoprim 1.25 µg / sulfamethoxazole 23.75 µg (SXT), aztreonam (ATM) 30 µg and imipenem (IPM) 10 µg.

Detection of antimicrobial resistance genes in CS6-associated ETEC

The choice of resistance genes studied was based on the high frequency of resistance to AMP and SXT (the most available inexpensive antimicrobial), observed previously [27]. CS6-expressing ETEC isolates were screened for the presence of $bla_{\text{TEM1,}}$ and two sulfonamides genes (*sulI* and *sulII*) by PCR (Table 1). Bacterial DNA extraction was performed as described elsewhere [28]. Oligonucleotide primers bla_{TEM}F and bla_{TEM}R (Table1) and the conditions necessary to amplify a 643 bp fragment were previously described [29]. Oligonucleotide primer pairs used were *sulI*F and *sulI*R or *sulII*F and s*ulII*R, following conditions described previously. Either a 433 bp or a 293 bp internal portion of the *sul1* or *sulII* genes (Table 1), respectively, was amplified [62].

Table 1. Characteristics of CS6-expressing ETEC isolates used in this study

ETEC-CF type	No of Isolates	ETEC-CF type	Toxin (gene)	Toxin type	O group	H group
CS6 isolates	6	CS6	ST (estA1)	STp	**169**	**41**
(n = 41)	1	CS6	ST (estA 2-4)	STh	**169**	**41**
	6	CS6	ST (estA1)	STp	**27**	**7**
	5	CS6	ST (estA1)	STp	**27**	**20**
	5	CS6	ST (estA1)	STp	**27**	**[a]NT**
	1	CS6	ST (estA1, eltB)	STp LT	**159**	**20**
	1	CS6	ST (estA1)	STp	**159**	**NT**
	1	CS6	ST (estA 2-4)	STh	**4**	**40**
	2	CS6	ST (estA 2-4)	STh	**4**	**NT**
	1	CS6	ST (estA1)	STp	**8**	**4**
	1	CS6	ST (estA1)	STp	**8**	**9**
	1	CS6	ST (estA1)	STp	**11**	**25**
	1	CS6	ST (estA 2-4)	STh	**25**	**NT**
	1	CS6	ST (no estA gene)	NT	**39**	**11**
	1	CS6	ST (estA 2-4)	STh	**78**	**18**
	1	CS6	ST (estA1)	STp	**148**	**28**
	1	CS6	ST (estA1)	STp	**157**	**NT**
	2	CS6	ST (estA1)	STp	**NT**	**NT**
	1	CS6	LT (no estA gene)	NT	**NT**	NT
	1	CS6	ST (estA1)	STp	**NT**	**9**
	1	CS6	LT (no eltB gene)	NT	**19**	**16**
CS6 & CS4 isolates	5	CS6 & CS4	STLT (estA1, eltB)	STp LT	**25**	**NT**
(n = 11)	1	CS6 & CS4	LTST (estA 2-4)	STh	**25**	**NT**
	1	CS6 & CS4	STLT (estA1, eltB)	STpLT	**[b]R**	**4**
	1	CS6 & CS4	STLT (estA1, eltB)	STpLT	**R**	**-**
	1	CS6 & CS4	STLT (estA1, eltB)	STpLT	**54**	**21**
	1	CS6 & CS4	STLT (estA1, eltB)	STpLT	**15**	**18**
	1	CS6 & CS4	ST (estA 2-4)	STh	**4**	**-**
CS6 & CS5 isolates	3	CS6 & CS5	STLT (estA 2-4,	STpLT	**39**	**12**
(n = 10)	1	CS6 & CS5	STLT(no estA gene)	NT	**39**	**12**
	1	CS6 & CS5	STLT (estA 2-4,	STpLT	**39**	**-**
	1	CS6 & CS5	STLT (estA 2-4,	STpLT	**167**	**-**
	1	CS6 & CS5	STLT (estA 2-4,	STpLT	**118**	**40**
	1	CS6 & CS5	ST (estA 2-4)	STh	**27**	**7**
	1	CS6 & CS5	ST (estA 2-4)	STh	**152**	**5**
	1	CS6 & CS5	ST (estA 2-4)	STh	**167**	**5**

Abbreviations: [a]NT: non-typeable; [b]R: rough colonies

Multiplex PCR for the detection of ETEC enterotoxins

Genes encoding the major structural subunit of known CS and the *elt*B1 (LT), *estA2* -4 (STh), *est*A1 (STp) toxin genes were detected by multiplex PCR (mPCR) using previously described conditions [30]. Bands of 133 bp, 239 bp or 402 bp indicated the amplification of *estA2* -4 (STh), *est*A1 (STp), and *elt*B1 (LT) respectively, and were amplified using oligonucleotide primers, estA2 -4F (aattgctactattcatgctttcaggac), estA2 - 4R (tctttttcacctttcgctcagg), STpF1 (atgaaaaagctaatgttggca), STpR1 (ttaataacatccagcacaggca), LThF1 (cataatgagtacttcgatagaggaac) and LThR1 (gaaacctgctaatctgtaaccatcc).

All PCR amplicons were subjected to electrophoresis through a 1% agarose gel (Bio-Rad Laboratories, Richmond, CA, USA) and molecular masses determined by comparison to a 100 bp DNA Ladder (Promega, Madison, WI, USA). *E. coli* ATCC 25922, a non-ETEC, antimicrobial-susceptible strain, was used as a negative control for each PCR assay. Gels were stained with ethidium bromide and visualized under UV light prior to digital image capture.

XbaI pulsetypes

XbaI macrorestriction profiling using pulsed-field gel electrophoresis of digested chromosomal DNA was used to detect genetic diversity among CS6-expressing isolates as described by PulseNet USA [31]. PFGE was performed using a CHEF DRII electrophoresis chamber (Bio-Rad); DNA was exposed to an electric current for 22 hours at 6 V/cm at 14ºC in 1% agarose gel by using a linear pulse ramp of 1.79 to 18.66 s in 0.5 X Tris-borate-EDTA electrophoresis buffer. PFGE profiles were compared by automated analysis using the BioNumerics software (version 5.10; Applied Maths, Austin, TX, USA) and confirmed by visual inspection. Similarity between PFGE banding patterns was calculated using the Dice coefficient with a 2% tolerance for the band migration distance and clustering was performed using the complete linkage method. This analysis method is considered a more strict interpretation of banding patterns than UPGMA [32].

Statistical analysis

Results were double-data entered into an Access database; descriptive statistics were completed using Epi Info version 6 (CDC, Atlanta, GA USA and WHO, Geneva, Switzerland). Chi-square or Fisher's exact tests were used to determine statistical significance among discrete or categorical variables.

Results

Phenotypic and genotypic characterization of antimicrobial resistance

Overall, resistance among all isolates in this study to AMP, SAM, TET and SXT was 47%, 7%, 24% and 37% respectively (Table 2). AMP resistance was comparable among ETEC isolates with CS6 alone (n = 19, 46%) and ETEC isolates co-expressing CS6 with either CS4 or CS5 (n = 10, 48%). SXT resistance was

Table 2. Antibiotic resistance of CS6 isolates

	Ampicillin (AMP)	**Ampicillin /sulbactam (SAM)**	**Tetracycline (TET)**	**Trimethoprim / Sulfamethoxisole (SXT)**
Isolates	number (%)	number (%)	number (%)	number (%)
CS6 expressed alone (n = 41)	19 (46)	2 (5)	8 (20)	13 (32)
CS6 co-expressed with CS4 or CS5 (n = 21)	10 (48)	2 (10)	7 (33)	10 (48)
All isolates (n = 62)	29 (47)	4 (7)	15 (24)	23 (37)

observed less frequently among ETEC isolates expressing CS6 alone (n = 13, 32%) compared to ETEC co-expressing CS6 with either CS4 or CS5 (n = 10, 48%); however, the difference was not statistically significant (P = 0.27). Similarly, TET resistance was lower among ETEC expressing CS6 alone (n = 8, 20%) compared with ETEC co-expressing CS6 with either CS4 or CS5 (n = 7, 33%), and this difference was not statistically significant due to the limited number of isolates available for examination.

Resistance of CS6-expressing ETEC- to AMP could be explained by the presence of TEM-1 ß-lactamases; bla_{TEM1} was detected in all CS6-expressing resistant isolates (n = 19). However, bla_{TEM1} was detected in only 50% (n = 5) of CS4 and CS6 or CS5 and CS6 co-expressing isolates that were AMP-resistant. Resistance to SXT was identified in 37% (n = 23/62) of ETEC isolates analyzed in this study. In total *sul1*-alone (Table 2) was detected least frequently (1/23), *sulII*-alone was more common (8/23), and detection of both genes was most common (10/23). Interestingly, CS6-expressing ETEC isolates were more likely to have both genes (9/13) whereas CS6 co-expressing CS4 or CS5 were more likely to have only the *sulII* gene (6/10). We were unable to detect *sulI* in CS6-coexpressing isolates; four isolates had no *sul* gene detected. All isolates were susceptible to TIM, FEP, CRO, CAZ, NAL, CIP, AN, GEN, ATM, IPM (data not shown).

Pulsetype toxin detection and serological characterization of CS6 isolates

Comparison of the results of PCR amplification of the toxin genes with previous phenotying data revealed six discrepant results (Table 3). Two of the isolates (a serotype O159:H20 isolate expressing CS6 alone and an O15:H18 isolate co-expressing CS6 and CS4) phenotypically expressed ST but were also found to contain the *eltB* gene encoding LT. Two isolates phenotypically LTST (O39:H11, CS6 alone and O39:H12, co-expressing CS6 and CS5) had no detectable gene encoding ST, and two isolates (rough, CS6 alone; O19:H16, CS6 alone) had no detectable gene encoding LT. Eight O-serotypes had multiple isolates; four O-serotypes (O4, 25, 27 and 39) contained isolates expressing different CS combinations. In each case, the unique CS expressing isolates also possessed different toxin genes with the exception of the CS4, CS6-co-expressing O4 isolate that appeared to share the same STh encoding gene. We also found diversity in toxin genes within isolates that had conserved sero- and CS types (O159, O167 and O169). However, the majority of ETEC isolates expressing CS6-alone (75%; n = 32) contained the *estA*1 gene, encoding for STp. Similarly, the majority of CS4, CS6 co-expressing isolates harboured *estA*1 (n = 9/11) in addition to the *eltB* gene. Only two CS4, CS6 co-expressing isolates were detected that had the *estA* 2-4 gene encoding for STh toxin. In contrast, the

Table 3. Carriage of β-lactam and sulfonamide resistance genes among CS6 isolates

Isolates	Phenotype of resistance	No. of isolates	Resistance gene detected	No. of isolates
CS6 expressed alone (n = 41)	AMP	19	bla_{TEM1}	19/ 19
	SXT	13	*sulI* only	1/ 13
			sul II only	2/ 13
			sulI and sulII	9/ 13
CS6 expressed with CS4 or CS5 (n = 21)	AMP	10	bla_{TEM1}	5/10
	SXT	10	*sulI* only	0
			sul II only	6/10
			sulI and sulII	1/ 10
All CS6 isolates (n = 62)	AMP	29	bla_{TEM1}	24/29
	SXT	23	*sulI* only	1/23
			sul II only	8/23
			sulI and sulII	10/23

majority of CS5, CS6 co-expressing isolates harboured an *estA*2-4 gene (9 /10); 6 of these isolates harboured *eltB*.

CS6 XbaI pulsetypes

XbaI digestion of whole genomic DNA extracted from CS6-expressing isolates revealed extensive band diversity (Figure 1A and 1B). There were 46 distinct XbaI-pulsetypes among the 62 CS6-expressing ETEC isolates. No common XbaI PFGE groups were observed when the pulsetypes for the CS6-expressing and the CS6, CS4 or CS5 co-expressing isolates were combined (data not shown). To facilitate comparisons, we analyzed CS6-expressing isolates separately from CS4 or CS5, CS6-co-expressing isolates.

Thirty-one pulsetypes were observed among the 41 CS6-expressing isolates (Figure 1A), although only 20 serotypes were detected in this same collection of isolates. Four broad clusters (I-IV) of isolates were observed among the isolates (33% similarity). Having a common O type was not an adequate indicator of XbaI pattern relatedness: O27 and O4 expressing isolates were located in three separate clusters; O8 and O159 isolates were found in two clusters. An exception to this was O169-expressing isolates; all seven isolates were located in cluster III. Isolates expressing the same O: H type, however, always grouped in the same cluster, with the exception of O4: H NT strains which grouped in separate clusters. The broad clusters consisted of discrete lineages at greater similarity values; these lines were often composed of isolates of the same serotype (e.g. O169: H41, O27: H NT, O27:H7, O27:H20). Isolates of O169: H41 (n = 7), the most common serotype among CS6-expressing ETEC isolates, had six unique pulsetypes. Six of the seven isolates formed a highly related branch in cluster III; the exceptional isolate clustered to a unique arm in cluster III. This isolate also differed from the other O169:H41 isolates in the ST allele expressed; WS6319 harboured an *estA2-4* allele whereas the remaining six isolates all had *estA1*.

Cluster II consisted of all of the O27 isolates, except O27:H20, in addition to the single O8:H4 isolate and one of the two O4:H NT isolates. The five O27:H20 isolates were split into cluster IV (n = 4) and cluster I (n = 1). All of the O27:H7 isolates expressed STp and were sensitive to all antimicrobial tested. The O27:H NT isolates clustered into a unique branch within cluster II, and all expressed STp but differed in their antimicrobial phenotypes. All the O27:H20 isolates expressed STp and had an identical antimicrobial resistance profile (AMP, SXT) and four of the five isolates were genetically indistinguishable (cluster IV). WS6607 was the sole O27:H20 isolate in cluster I, with a genetic background distinct from other O27:H20 isolates.

XbaI pulsetypes of ETEC isolates co-expressing CS6 with CS4 or CS5

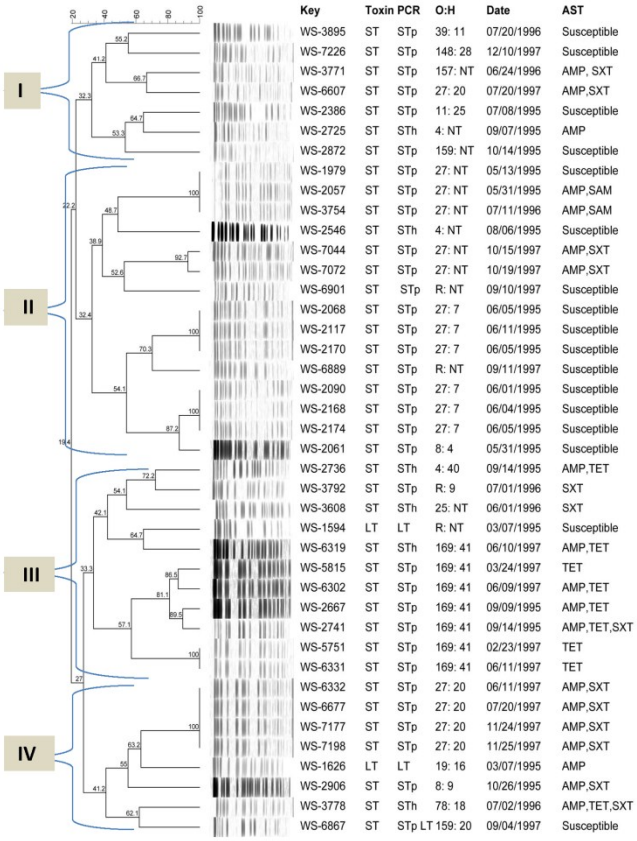
We observed 15 XbaI pulsetypes with CS6- ETEC isolates co-expressing CS4 or CS5 (Figure 1B). All of the CS4, CS6 co-expressing isolates grouped together at a similarity level of 60% and were clearly distinct from the CS5, CS6 isolates. Three distinct lineages were observed emerging from within the CS5, CS6 co-expressing isolates. One branch had an O167 isolate; the second branch consisted exclusively of O39-expressing isolates; and the last branch was diverse, containing an O167 isolate, two unique serotypes as well as the sole O27:H7 isolate that co-expressed CS5 and CS6. This last isolate also differed from all the other O27 isolates as it expressed STh. All of the CS5, CS6 co-expressing isolates secreted STh.

Among the eleven isolates that co-expressed CS4 and CS6, six had an indistinguishable XbaI pulsetype, expressed LT and ST, and were serotype O25: NT (Figure 1B). Five of the six isolates secreted STp, while one isolate, WS2577, expressed STh. Four of the six isolates were susceptible to all antimicrobials tested in this study and two were SXT resistant. The six XbaI PFGE indistinguishable isolates were all recovered in August 1995 from six different children in two villages in Abu Homos. This cluster might represent an outbreak among the children in Abu Homos; unfortunately, it was not possible to confirm this in our study. The remaining CS4, CS6 co-expressing isolates differed in PFGE profile, antimicrobial susceptibility pattern, and toxin profile.

Discussion

Antimicrobial-resistant ETEC are a growing public health concern. While treatment of diarrhoea with antimicrobial agents is generally restricted to severe cases or the immunocompromised, at least one travel clinic has reported that 35% of returning travellers have been treated with an antimicrobial due to disease severity or duration [57]. Antimicrobial resistance profiles of 3,627 isolates of ETEC and non-enterotoxigenic *E. coli* recovered during 1995-2000 from infants and children residing in the Abu Homos district, Egypt, have previously been examined [27]. The percentage of antimicrobial resistant ETEC isolates was 64.6% for ampicillin, 53.8% for

Figure 1A. Dendrogram and characteristics of ETEC expressing CS6 alone based on XbaI-digested genomic DNA.

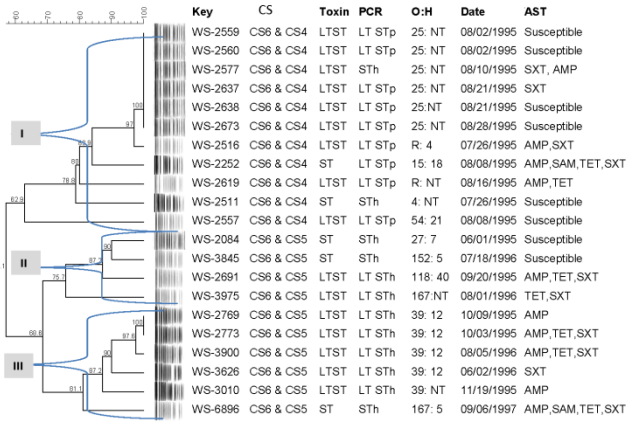


Key	Toxin	PCR	O:H	Date	AST
WS-3895	ST	STp	39: 11	07/20/1996	Susceptible
WS-7226	ST	STp	148: 28	12/10/1997	Susceptible
WS-3771	ST	STp	157: NT	06/24/1996	AMP, SXT
WS-6607	ST	STp	27: 20	07/20/1997	AMP,SXT
WS-2386	ST	STp	11: 25	07/08/1995	Susceptible
WS-2725	ST	STh	4: NT	09/07/1995	AMP
WS-2872	ST	STp	159: NT	10/14/1995	Susceptible
WS-1979	ST	STp	27: NT	05/13/1995	Susceptible
WS-2057	ST	STp	27: NT	05/31/1995	AMP,SAM
WS-3754	ST	STp	27: NT	07/11/1996	AMP,SAM
WS-2546	ST	STh	4: NT	08/06/1995	Susceptible
WS-7044	ST	STp	27: NT	10/15/1997	AMP,SXT
WS-7072	ST	STp	27: NT	10/19/1997	AMP,SXT
WS-6901	ST	STp	R: NT	09/10/1997	Susceptible
WS-2068	ST	STp	27: 7	06/05/1995	Susceptible
WS-2117	ST	STp	27: 7	06/11/1995	Susceptible
WS-2170	ST	STp	27: 7	06/05/1995	Susceptible
WS-6889	ST	STp	R: NT	09/11/1997	Susceptible
WS-2090	ST	STp	27: 7	06/01/1995	Susceptible
WS-2168	ST	STp	27: 7	06/04/1995	Susceptible
WS-2174	ST	STp	27: 7	06/05/1995	Susceptible
WS-2061	ST	STp	8: 4	05/31/1995	Susceptible
WS-2736	ST	STh	4: 40	09/14/1995	AMP,TET
WS-3792	ST	STp	R: 9	07/01/1996	SXT
WS-3608	ST	STh	25: NT	06/01/1996	SXT
WS-1594	LT	LT	R: NT	03/07/1995	Susceptible
WS-6319	ST	STh	169: 41	06/10/1997	AMP,TET
WS-5815	ST	STp	169: 41	03/24/1997	TET
WS-6302	ST	STp	169: 41	06/09/1997	AMP,TET
WS-2667	ST	STp	169: 41	09/09/1995	AMP,TET
WS-2741	ST	STp	169: 41	09/14/1995	AMP,TET,SXT
WS-5751	ST	STp	169: 41	02/23/1997	TET
WS-6331	ST	STp	169: 41	06/11/1997	TET
WS-6332	ST	STp	27: 20	06/11/1997	AMP,SXT
WS-6677	ST	STp	27: 20	07/20/1997	AMP,SXT
WS-7177	ST	STp	27: 20	11/24/1997	AMP,SXT
WS-7198	ST	STp	27: 20	11/25/1997	AMP,SXT
WS-1626	LT	LT	19: 16	03/07/1995	AMP
WS-2906	ST	STp	8: 9	10/26/1995	AMP,SXT
WS-3778	ST	STh	78: 18	07/02/1996	AMP,TET,SXT
WS-6867	ST	STp LT	159: 20	09/04/1997	Susceptible

The scale at the top of the figure represents percentage similarity, with 100 as indistinguishable and values decreasing to 0 increasingly less similar, percentage similarity at branch nodes are indicated.

Abbreviations: Key: strain designation; Toxin: expression of either the heat stable toxin (ST) or heat-labile toxin (LT); PCR: detection of either the *estA1* gene (STp), *estA2-4* genes (STh), or *eltB* gene (LT); Date: date of isolation at the US Naval Medical Research Unit No. 3, Cairo, Egypt (NAMRU-3) laboratory; AST: results of antimicrobial testing with resistance to ampicillin (AMP), sulfatrimethoxazole (SXT), tetracycline (TET) or fully susceptible indicated.

Figure 1B. Dendrogram and characteristics of ETEC expressing CS6 co-expressing either CS4 or CS5 based on XbaI-digested genomic DNA



Key	CS	Toxin	PCR	O:H	Date	AST
WS-2559	CS6 & CS4	LTST	LT STp	25: NT	08/02/1995	Susceptible
WS-2560	CS6 & CS4	LTST	LT STp	25: NT	08/02/1995	Susceptible
WS-2577	CS6 & CS4	LTST	STh	25: NT	08/10/1995	SXT, AMP
WS-2637	CS6 & CS4	LTST	LT STp	25: NT	08/21/1995	SXT
WS-2638	CS6 & CS4	LTST	LT STp	25:NT	08/21/1995	Susceptible
WS-2673	CS6 & CS4	LTST	LT STp	25: NT	08/28/1995	Susceptible
WS-2516	CS6 & CS4	LTST	LT STp	R: 4	07/26/1995	AMP,SXT
WS-2252	CS6 & CS4	ST	LT STp	15: 18	08/08/1995	AMP,SAM,TET,SXT
WS-2619	CS6 & CS4	LTST	LT STp	R: NT	08/16/1995	AMP,TET
WS-2511	CS6 & CS4	ST	STh	4: NT	07/26/1995	Susceptible
WS-2557	CS6 & CS4	LTST	LT STp	54: 21	08/08/1995	Susceptible
WS-2084	CS6 & CS5	ST	STh	27: 7	06/01/1995	Susceptible
WS-3845	CS6 & CS5	ST	STh	152: 5	07/18/1996	Susceptible
WS-2691	CS6 & CS5	LTST	LT STh	118: 40	09/20/1995	AMP,TET,SXT
WS-3975	CS6 & CS5	LTST	LT STh	167:NT	08/01/1996	TET,SXT
WS-2769	CS6 & CS5	LTST	LT STh	39: 12	10/09/1995	AMP
WS-2773	CS6 & CS5	LTST	LT STh	39: 12	10/03/1995	AMP,TET,SXT
WS-3900	CS6 & CS5	LTST	LT STh	39: 12	08/05/1996	AMP,TET,SXT
WS-3626	CS6 & CS5	LTST	LT STh	39: 12	06/02/1996	SXT
WS-3010	CS6 & CS5	LTST	LT STh	39: NT	11/19/1995	AMP
WS-6896	CS6 & CS5	ST	STh	167: 5	09/06/1997	AMP,SAM,TET,SXT

The scale at the top of the figure represents percentage similarity, with 100 as indistinguishable and values decreasing to 0 increasing less similar, percentage similarity at branch nodes are indicated.

Abbreviations: strain designation; CS: coli surface antigen(s) detected; Toxin: expression of either the heat stable toxin (ST), heat-labile toxin (LT) or both toxins (LTST); PCR: detection of either the *estA1* gene (STp), *estA2-4* genes (STh), or *eltB* gene (LT) or a combination of the genes; Date: date of isolation at the US Naval Medical Research Unit No. 3, Cairo, Egypt (NAMRU-3) laboratory; AST: results of antimicrobial testing with resistance to ampicillin (AMP), AMP-sulbactam (SAM), trimethoprim-sulfatrimethoxazole (SXT), tetracycline (TET) or fully susceptible as indicated.

trimethoprim-sulfamethoxazole, and 41.5% for tetracycline. Overall, the ETEC isolates examined in this study were sensitive to the majority of the antimicrobials tested. Resistance to ampicillin was observed in nearly half of the isolates, and in most cases, this resistance could be explained by acquisition of a member of the bla_{TEM1} family. Unlike previous studies conducted in Egypt, observed resistance in this study is consistent with ETEC resistance reported in India [57] and Vietnam [58], but less than that observed in Thailand [59]. Our data also revealed the presence of resistance genes *sul1* (n = 1, 4%) *sulII* (n = 8, 35%) and both genes (n = 10, 43%) among trimethoprim-sulfamethoxazole resistant isolates. We observed that *sulI* and *sulII* resistance tended to be associated more often with CS6-expressors (n = 9, 69%), whereas *sulII* alone was more commonly associated with CS4 or CS5, CS6 expressing isolates (n = 6, 60%).

Correlation of observed antimicrobial resistance to expressed colonization factors has not previously been performed in Egypt and it is possible that ETEC expressing colonization factors other than CS6 are more resistant to ampicillin and trimethoprim-sulfamethoxazole. Detection of antimicrobial resistance mechanisms for ampicillin and sulfa drug resistance should provide a reference point for studies of emerging resistance in ETEC isolated from Egypt.

Diversity in toxin type and serotype of ETEC isolates expressing CS6 has been previously reported. Our results also suggest that CS6 antigen is expressed in a wide representation of phenotypically and genetically dissimilar ETEC isolates, supporting the use of CS6 antigen as a vaccine candidate to control ETEC infections. In this study 16 of 17 (94%) O27-expressing isolates expressed only CS6. McConnell *et al*. [42] also reported that 94% and 86% of O25- and O27-expressing isolates were found to express CS6 alone. McConnell *et al*. were the first to suggest that CS6 might be able to function as a colonization factor, independent of an association with either CS4 or CS5.

The majority of the O25 isolates described in the present study secreted LT and ST and co-expressed CS4 with CS6 (6/7; 86%); this observation is in contrast to other studies where O25 isolates were LT-only. Serological analysis of 108 CS6-associated ETEC isolates (79 expressing CS6-only and 29 co-expressing either CS4 or CS5) revealed 33 distinct O: H serotypes: 20 distinct O: H serotypes among the ETEC isolates expressing CS6 alone and 13 O: H serotypes within the CS4 or CS5 co-expressing isolates [8]. The most common serologic feature among CS6-expressing isolates was detection of the O27 lipopolysaccharide [O27: H7 (n = 6), O27: H20 (n = 5) or O27: H NT (n = 5)], while the most common serotype detected was O169: H41 (n = 7). However, because genetically unrelated strains may express the same phenotypic markers [43] and genetically related strains may express traits differently, phenotypic testing has a restricted ability to discriminate between strains. More recently, several groups have reported nucleotide heterogeneity in the operon containing the genes encoding CS6 [23, 44], supporting the concept of genetic diversity within CS6 expressing isolates. Since genes that code for toxins and CS usually are located in plasmids, these markers are not likely reliable markers of clonality of ETEC [18].

DNA-based techniques such as PFGE have been used for the characterization of diarrhoeal pathogens, including ETEC [45,46]. PFGE is recognized as a useful tool for comparative analyses of bacterial isolates, but genomic inferences may be difficult to support without additional restriction enzyme analyses [47].

Multilocus sequence analysis (MLST) of a global collection of ETEC isolates, including some strains from Egypt, indicate that there are at least 42 lineages [51]. Consistent with our findings, strains of CS6 populate 15 of these lineages, indicating widespread genomic heterogeneity. PFGE has also been shown to be useful for outbreak [52,53] and non-outbreak/surveillance of *E. coli* [54, 55]. In this study we selected PFGE as the method for phylogenetic analysis, in part to create a database of ETEC isolates that would form the basis of the PulseNet Middle East *E. coli* system. Noller *et al*. [56] have previously shown that strain diversity in one serotype of *E. coli* may be underestimated using MLST. Current studies are underway evaluating the diversity of our CS6-expressing ETEC isolates using both methods.

In conclusion this study has used PFGE to investigate the diversity of CS6 expressing ETEC in Egypt. Our observations, based on PFGE analysis, suggest a high degree of heterogeneity among CS6 strains in this region, indicating a non-clonal origin and revealing intra-serotype variation. However, CS6 isolates that express the same O: H serotype, such as O27: H7 and O27: H20, appear clonally related. Implementation of standardized protocols of DNA-based typing methods will yield a PFGE database of ETEC strains that could improve the analysis of data generated at different locations and could contribute to a better understanding of the genetic structure of ETEC strains.

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Disclaimer

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