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Multilocus genotypic characterization of *Escherichia coli* O157:H7 recovered from food sources

M. M. ELHADIDY[1]* AND W. F. ELKHATIB[2,3]

[1] *Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt*
[2] *Department of Microbiology & Immunology, Faculty of Pharmacy, Ain Shams University, African Union Organization, St. Abbassia, Cairo, Egypt*
[3] *Department of Pharmacy Practice, School of Pharmacy, Hampton University, Hampton, VA, USA*

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SUMMARY

Escherichia coli O157:H7 strains ($n = 33$) recovered from different food sources in Egypt were characterized using molecular assays to identify strain genotypes associated with various levels of pathogenic potential. Genotypic characterization included: lineage-specific polymorphism assay (LSPA-6), Shiga-toxin-encoding bacteriophage insertion site assay (SBI), clade 8 typing, *Tir* (A255 T) polymorphism, and variant analysis of Shiga toxin 2 gene (Stx_{2a} and Stx_{2c}), and anti-terminator *Q* genes (Q_{933} and Q_{21}). Genotypes LI/II (76%), SBI 1 (60·6%), clade 8 (69·7%), *Tir* (255 T) (72·7%) and Stx_{2c} (45·5%) were found to be significantly more frequent compared to other genetic markers in the strains analysed. Multivariable analysis revealed a significant association between LPSA-6 and clade types as well as *Tir* (A255 T). To the best of our knowledge, this is the first study to report the characterization of these genetic markers in *E. coli* O157:H7 strains in the Middle East and Africa.

Key words: Characterization, *E. coli* O157:H7, food, genotypes.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is an important foodborne pathogen of global significance that represents a serious public health hazard and financial burden [1]. Cattle are the primary reservoir of *E. coli* O157:H7 and transmission to humans occurs through the consumption of food or water contaminated with cattle faeces, contact with infected animals or their environment, and by person-to-person contact [2]. Symptoms of infection range from subclinical to bloody diarrhoea, vomiting, haemorrhagic colitis, and life-threatening sequelae, such as haemolytic uraemic syndrome (HUS) [2].

Several epidemiological studies have characterized *E. coli* O157:H7 strains into genotypes associated with altered pathogenic potential and host specificity [3–8]. The identification of such virulent genotypes is crucial to improve the understanding of *E. coli* O157:H7 pathogenesis and facilitate development of new strategies for its prevention and control [6, 9]. These assays include the lineage-specific polymorphism assay (LSPA-6) [1, 10, 11], Shiga toxin bacteriophage insertion (SBI) site [12, 13], clade 8 typing [6], *Tir* (A255 T) polymorphism [6], Shiga toxin 2 variants (Stx_{2a} and Stx_{2c}), and Sx_2-*Q* anti-terminator Q_{933}/Q_{21} gene variants [3,7].

The aim of this study was to characterize *E. coli* O157:H7 strains recovered from food sources in

* Author for correspondence: Dr M. M. Elhadidy, Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.
(Email address: mm_elhadidy@mans.edu.eg)

Egypt using molecular subtyping methods to identify genotypes associated with varying levels of virulence potential. Consequently, discrimination of these genotypes may permit tracking and prediction of highly pathogenic strains directly from food sources.

MATERIALS AND METHODS

Bacterial strains

Thirty-three *E. coli* O157:H7 strains isolated from meat and dairy food sources were collected from different localities in Egypt during the period April 2012–February 2013. The meat samples ($n = 215$) included 75 retail minced beef, 70 hamburger, and 70 fresh beef samples; dairy samples ($n = 215$) included 100 raw milk and 115 raw milk cheese samples (60 Kareish cheese, 55 Domiati cheese). All samples were collected at random from different supermarkets, butchers, retail and dairy shops in two governorates (Cairo and Gizah) in Egypt. Microbiological screening and confirmation of *E. coli* O157:H7 was performed as previously described [14]. Isolates were grown on tryptone soy agar (TSA; Oxoid Ltd, UK) and incubated aerobically at 37 °C for 24 h. Bacterial DNA was purified using DNeasy Blood & Tissue kit (Qiagen, USA). All isolates possessed either the gene encoding Shiga toxin 1 (Stx_1) or Shiga toxin 2 (Stx_2) or both, as well as intimin (*eae*) and enterohaemolysin (*ehx*) genes.

Pulsed-field gel electrophoresis (PFGE)

The study strains were analysed by PFGE to confirm non-clonality using *Xba*I restriction according to the PulseNet protocol from the Centers for Disease Control and Prevention [15]. At least one band difference was required to distinguish between pulsotypes.

LSPA-6 genotype

LSPA-6 genotyping was performed in two multiplex PCR reactions [11] using primers and cycling conditions as previously described [8].

Tir polymorphism

Single nucleotide polymorphisms (A/T) located in the *Tir* gene were detected by a probe-based real-time PCR as published previously [5].

Clade 8 typing

Clade 8 strains were identified using hairpin primers and cycling conditions as described previously [16] and results were analysed with LightCycler® 480 (Roche Diagnostics, USA) using LightCycler 480 software.

SBI genotyping

SBI genotypes of strains were determined according to previous studies [4, 17] with minor modifications. Amplification of *yehV* or *wrbA* was performed in two multiplex PCR reactions for detection of bacteriophage integration using published primers [17], while amplification of Stx_1 and Stx_2 genes was performed in two uniplex reactions [18] and PCR amplicons were coded and assigned as described previously [19].

Stx_2 and Sx_2-Q anti-terminator gene variants

Detection of Stx_{2a} and Stx_{2c} variants, and bacteriophage anti-terminator gene alleles (Q_{933} and Q_{21}) was carried out by their respective PCR [3, 20].

Statistical analyses

Genotype data were analysed by two-tailed non-parametric Mann–Whitney test and $P < 0{\cdot}05$ was considered statistically significant. Statistical analyses were performed with SPSS v. 18·0 (SPSS Inc., USA).

RESULTS AND DISCUSSION

E. coli O157:H7 was detected in 7·6% of the food samples analysed overall with frequencies of 8·4% (18/215) from meat products and 6·9% (15/215) from dairy products. This finding contrasts with the lower rate (3·1% in meat and 3·6% in dairy products) found in a recent large-scale survey of *E. coli* O157:H7 from food samples (800 meat and 800 dairy products) in different cities and villages in Egypt [21]. This difference between the surveys might be attributed, in part, to differences in study methodology such as sampling strategy, type of samples, enrichment procedures, immunomagnetic separation and culture media [22]. In meat products, *E. coli* O157:H7 was detected at a higher rate in minced beef samples (12%), followed by hamburgers (7·1%), and fresh beef meat (5·7%), whereas in dairy samples, detection rates were highest in raw milk cheese samples (7·8%), followed by raw milk samples (6%).

Earlier studies have reported the importance of the molecular assays used in this study in identifying *E. coli* O157:H7 genotypes with variation in pathogenic potential and host specificity [3–8]. Furthermore, such assays were proposed as valuable indicators for

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Table 1. *Distribution of different genotypes in* E. coli *O157:H7 isolates recovered from food sources in Egypt*

Assay	Genotypes	Frequency of distribution
LSPA-6	LI	1 (3%)
	LI/II	25 (76%)
	LII	7 (21%)
SBI	1	20 (60·6%)
	3	1 (3%)
	5	6 (18·2%)
	6	5 (15·2%)
	21	1 (3%)
Clade 8 typing	Clade 8	23 (69·7%)
	Non-clade 8	10 (30·3%)
Tir (A255 T)	*Tir* 255 T	24 (72·7%)
	Tir 255A	9 (27·3%)
Q_{21}–Q_{933}	Q_{21}	16 (48·5%)
	Q_{933}	15 (45·5%)
	$Q_{933+}Q_{21}$	2 (6%)
Stx_{2a}/Stx_{2c}	Stx_{2a}	11 (33·3%)
	Stx_{2c}	15 (45·5%)
	Stx_{2a+c}	7 (21·2%)

LSPA-6, Lineage-specific polymorphism; SBI, Shiga-toxin-encoding bacteriophage insertion site.

countries with high rates of outbreak investigations with this organism [23]. The LSPA-6 assay detects differences in six loci representing conserved regions of the O157 backbone that differentiate *E. coli* O157:H7 strains into three genotypes referred to as 'lineages' (LI, LI/II, LII) [11, 24]. Table 1 shows that of the 33 *E. coli* O157:H7 strains investigated here 25 (76%) were of lineage I/II, seven (21%) strains were lineage II and only one strain was lineage I. Previous studies indicate that the predominant lineage I/II shares characteristics of both lineage I and lineage II, but in contrast to lineage II, it is associated with human illness at frequencies similar to those of lineage I strain, and includes hypervirulent strains recovered from a multistate 'spinach' outbreak in Canada [10, 11]. Moreover, lineage I/II strains have been correlated with severe human disease outcomes such as HUS [25]. The current study provides further evidence of the epidemiological relevance of lineage I/II as a possible emerging risk genotype in many countries and possibly Egypt. On the other hand, a different LSPA-6 distribution profile (lineage II: 48·3%, lineage I/II: 37·9%, lineage I: 13·8%) was reported in *E. coli* O157:H7 from food sources in The Netherlands and such frequencies exhibited an intermediate distribution of lineage I/II between bovine and human clinical strains, suggesting a possible role of food in the selection of potentially virulent genotypes in humans [26]. This divergence in LSPA-6 distribution may be attributed to geographical differences and disease incidence in different countries, as well as the environmental load originating from super-shedding of *E. coli* O157:H7 by cattle [26, 27].

Differentiation of SBI genotypes relies on amplification of the toxin genes Stx_1 and Stx_2 and the insertion site junctions of their encoding bacteriophages. This assay has been used to categorize *E. coli* O157:H7 isolates based on their propensity to cause disease in humans and some SBI genotypes (1, 2, 3) were found to be clinically biased genotypes [4, 17]. In the strains studied here five different SBI genotypes were identified (1, 3, 5, 6, 21) (Table 1) and genotype 1 (60·6%) was significantly more prevalent ($P < 0·05$) which may provide initial evidence of its epidemiological significance as an indicator of a potential infection risk from food sources in Egypt. This finding is consistent with former studies that described SBI genotype 1 as a potential risk genotype in the USA [4] and The Netherlands [26]. SBI 5 and 6 accounted for fewer strains (18·2% and 15·2%, respectively) while SBI 3 and 21 were infrequent (3%) suggesting the possible limited relevance of these genotypes in Egypt.

Another subtyping scheme based on 32 single nucleotide polymorphisms was recently developed that could distinguish *E. coli* O157:H7 strains into nine distinct evolutionary clades, of which clade 8 was more frequently associated with severe disease outcomes such as HUS than other clades [6]. The majority (69·7%) of the strains here were typed as clade 8 (Table 1).

The gene *tir* encodes the translocated intimin receptor that mediates adhesion of *E. coli* to mammalian cells and the formation of attaching and effacing (A/E) lesions through binding to intimin [28]. The *Tir* (A255 T) polymorphism assay identifies a base change (A or T allele) at position 255 in the sequence of the gene and the variant *Tir* (255 T) has been significantly more associated with human disease than *Tir* (255A) [5]. Furthermore, most (62·1%) of *E. coli* O157:H7 isolates recovered from food sources in The Netherlands demonstrated the *Tir* (255 T) allele [26]. Congruent to this finding, our results revealed that *Tir* (255 T) was significantly more frequent ($P < 0·05$) in the tested *E. coli* O157:H7 isolates with 24 isolates (72·7%) exhibiting the T allele (Table 1). This observation may suggest a possible link of *Tir* (255 T) with increased risk of human infection with *E. coli* O157:H7 strains from food sources in Egypt.

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Table 2. *Multivariate analysis of genotypes in* E. coli *O157:H7 isolated from food sources in Egypt*

Source	Variables	Type III Σ squares	D.F.	*F*	Sig.	Partial η^2	Noncentrality parameter	Observed power
LSPA-6 genotypes	Q_{21}/Q_{933}	3·021	2	5·012	0·013	0·250	10·024	0·773
	Stx_{2a}/Stx_{2c}	1·298	2	1·201	0·315	0·074	2·401	0·242
	SBI genotypes	14·528	2	0·476	0·626	0·031	0·952	0·121
	Tir (A255 T)	4·705	2	38·360	**0·000**	0·719	76·719	**1·000**
	Clade types	251·355	2	41·818	**0·000**	0·736	83·636	**1·000**

LSPA-6, Lineage-specific polymorphism.
Significant values appear in bold.

The final characterization of the *E. coli* O157:H7 isolates included variants of the Stx_2-*Q* anti-terminator junction alleles (Q_{933} and Q_{21}) [29] as well as Stx_2 gene variants [30–32]. The Q_{933} variant which is located upstream of the Stx_2 gene and results in relatively high expression of the latter, was initially reported as a possible human infection risk indicator [33]. We found an almost equal distribution of Q_{933} and Q_{21} gene variants (45·5% and 48·5%, respectively) in Egyptian strains and two strains carried both gene variants (Table 1). Analysis of Stx_2 variants (Stx_{2a} and Stx_{2c}) based on phage chromosomal integration site, gene content and lineage placement [34] revealed that 11 (33·3%) strains carried Stx_{2a}, 15 (45·5%) strains carried Stx_{2c} and the remaining seven strains carried both variants (Stx_{2a+c}) (Table 1). Stx_{2c} variants have been shown to elicit milder symptoms and be less clinically significant, as well exhibiting less virulence in human kidney cell lines and mouse infection models than Stx_{2a} variants [7, 30, 35, 36].

Further analysis of correlations between the various genotyping methods showed that all clade 8 isolates and the majority (95·8%) of *Tir* (255 T)-carrying strains were represented by lineage I/II. By contrast, 70% of non-clade 8 and *Tir* (255A) (77·8%) carrying strains were grouped in lineage II. However, *Tir* (255 T) and *Tir* (255A) variants were more frequent in clade 8 (87·5%) and non-clade 8 (66·7%) strains, respectively. Last, most Q_{933} variants (93·3%) were associated with SBI 1, but Q_{21} variants grouped in different SBI genotypes [SBI 5 (37·5%), SBI 6 (31·25%), SBI 4 (25%), SBI 1 (6·25%)]. Multivariate analysis of different genotypes revealed evidence of a significant association between LSPA-6 and clade types as well as *Tir* (A255 T) genotypes at a confidence interval of 95% and a significance level of $P < 0·001$ (Table 2). This suggested that clade types and *Tir* (A255 T) polymorphism might be used not only as useful indicators for microbial risk potential by *E. coli* O157:H7 from food sources but also as possible surrogate markers of predictive of LSPA-6 genotypes. This strong association between clade 8 and lineage I/II is consistent with similar results from previous studies [24, 37, 38]. Consequently, such findings suggest that within lineage I/II, clade 8 strains exhibit higher virulence and develop more severe clinical diseases compared to other clades [6]. Conversely, no specific association between clade 8 and LSPA-6 genotypes was observed in *E. coli* O157:H7 strains recovered from bovine, food, and human sources in The Netherlands [26]. Nevertheless, a strong correlation between LSPA-6 lineage and *Tir* (A255 T) was previously reported in *E. coli* O157:H7 strains recovered from super-shedding cattle in USA [39] as well as in bovine and human *E. coli* O157:H7 strains isolated in the United States and Australia [23].

In conclusion, characterization of *E. coli* O157:H7 strains recovered from different food sources in Egypt revealed evidence of a higher frequency of distribution of lineage I/II, SBI 1, clade 8, *Tir* (255 T), and Stx_{2c} compared to other genotypes. Consequently, these genotypes might be utilized as potential valuable indicators of human infection risk from food sources. While our study was limited to a relatively small number of *E. coli* O157:H7 isolates, the findings may contribute to the international monitoring of infection risk genotypes that represent high virulence potential. To the best of our knowledge, there are no published data on the frequency of *E. coli* O157:H7 from human infections in Egypt and as a consequence such studies are urgently required to extend our analysis to determine the clinical incidence of such infections in Egypt and to determine the relationship between the described genetic markers, disease incidence, and clinical outcomes.

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DECLARATION OF INTEREST

None.

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