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ORIGINAL ARTICLE

Phenotypic and genotypic characterization of *Escherichia Coli* O111 serotypes

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ABSTRACT

Aim: The purpose of this study was to characterize phenotypically and genotypically the serotypes of the *E. coli* O111 associated with diarrheal disease and assess the variation among serotypes in terms of specific virulence factors and HeLa cells adherence patterns.

Background: *Escherichia coli* O111 serogroups are prevalent in endemic or sporadic cases of diarrhea, especially in developing areas.

Patients and methods: A total of 54 strains of *E. coli* O111 isolated from diarrheal and healthy cases were included in this study. Flagella antigens of motile and non-motile strains were identified by *fliC*-RFLP method (H types) and confirmed with agglutination test using H-specific antisera. All strains were tested for the presence of 5 different gene regions associated with virulence (*eaeA, eaeB, bfpA*, sxt and EAF plasmid) by PCR and the patterns of bacterial attachment to HeLa cells was assayed in cell culture.

Results: Of 54 *E. coli* O111 strains, 89% were typeable with standard H antisera and the remaining 11% of strains were non-motile (H -). Twenty-three different H type were distinguished among the O111 strains by PCR-RFLP. The most common serotypes included H21, H9, H2, H6 and H12 (48%). Serotypes O111:H9 were represented by strains with 2 patterns of virulence genes (*eaeA*$^+$*/bfpA*$^+$/EAE$^+$, and *eaeA*$^+$*/bfpA*$^-$/EAE$^-$*)* and serotype H14 was represented by strains with the single *eaeA*$^+$*/bfpA*$^+$/EAE$^-$ combination. Four distinct patterns of adherence were distinguished: LA, LLA, AA and DA. All of serotypes with the *eaeA*$^+$*/bfpA*$^+$/EAE$^+$, or eaeA$^+$/bfpA$^+$/EAE$^-$, combination isolated from children with diarrhea exhibited the LA pattern, and serotypes with eaeA$^+$/bfpA$^-$/EAF$^-$ showed the LLA, while the majority of the strains isolated from healthy cases exhibited the DA, AA and NA patterns.

Conclusion: Strains of this O serogroup represented a diverse of serotypes with a variety of virulence factors and mechanisms of pathogenesis.

Keywords: *E. coli*, Adherence pattern, Virulence properties, Serotype O111, Diarrhea.

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Introduction

E. coli is the major inhabitant of human gastrointestinal tracts and considered as non pathogen. However, specific strains of *E. coli* can cause different disease such as urinary tract infections, meningitis, wound infections, bacteremia, and diarrhea in human. The *E. coli* strains that cause diarrhea are considered as diarrheagenic *E. coli* (1-3), and belonged to certain

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serotypes. Six types of diarrheagenic *E. coli* (DEC) have been identified according to their serotypes and virulence factors including enterotoxigenic *E. coli* (ETEC), shiga-toxin producing *E. coli* (STEC) or enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffuse adherent *E. coli* (DAEC) (4). Each type of DEC includes different O and H antigens.

E. coli O111 strains were the first serogroup identified as a cause of severe outbreaks of infantile diarrhoea in nurseries in the United Kingdom in the 1940s. These strains are also prevalent in sporadic diarrhea cases. *E. coli* O111 serotypes causes enteropathogenic and enterohemorrhagic diseases in humans (5). EPEC O111 serogroup is a classic agent of diarrhea in children, especially in the developing areas. EHEC O111, which carries Shiga toxin genes, is one of the most common non O157 *E. coli* causes of bloody diarrhea and hemolytic uremic syndrome (HUS) in different areas of the world (6-10). Many outbreaks have been implicated to this pathogen (8, 9, 11-12).

Studies on O111 strains of different origin have shown that there is a considerable genetic and phenotypic heterogeneity among this *E. coli* O serogroup. Although motile O111 strains isolated from outbreaks of infantile diarrhea usually have H2, H12, or H21 flagella antigens, but in many cases non-motile (H^-) isolates are seen. Strains with serotypes O111:H2 and O111:H- typically carry the EAF plasmid that mediates localized adherence (LA) of bacteria to cultured cells that is a characteristic of the classic EPEC serotypes. The purpose of this study was to identify the major serotypes of the *E. coli* O111 serogroup associated with diarrhea disease in the study region and assess the variation among serotypes with regard to specific virulence factors, such as *eaeA, eaeB, bfpA, EAF, stx* genes and HeLa cells adherence patterns.

Patients and Methods

The isolates used in this study were originally recovered from patients with diarrhea and healthy cases. A total of 54 *E. coli* O111 isolates (2003-2004) were investigated. Of these, 36 isolates collected from children with enteric disease, and 18 from patient without symptom.

Determination of O antigen was performed as described (13) by agglutination tests using monospecific O111 antisera and H-types were determined with H-specific antisera (H1-H56) according to the instructions of the manufacturer (Bio-Rad Co and Statens Serum institute, Copenhagen, Denmark, respectively).

E. coli: O111 strains were cultured in LB broth media and DNA was extracted by boiling method. Flagella (*fliC*) genotypes of motile and non-motile O111 strains were identified by PCR followed by digestion of PCR products with *Hha*I restriction enzyme as described previously (14), and confirmed with agglutination test using H antigen specific antisera. The O111 isolates were also investigated for the presence of *eaeA, eaeB, bfpA, EAF*, and the production of Shiga toxins (Stx) as virulence genes by PCR. Primers used for PCR amplification are shown in table 1 (15-19).

Prototype EPEC strain E2348/69 (serotype O127: H6), which expressed BFP, EAF, intimin (20), and *E. coli* EDL933 which expressed Shiga toxins were used as positive control and *E. coli* K12: HB101 as a negative control in all assays.

Adherence to HeLa cells was investigated as described by Scaletsky et al (21). *E. coli* strains without adherence after 3 hours of incubation were submitted to a 6 hour adherence test. D-mannose (1%) was added to the medium to inhibit adherence through mannose-sensitive pilli. EPEC strain E2348/69 (serotype O127: H6) and *E. coli* K12 toxins were used as positive control (LA phenotype) and negative control (non –Adherence) in this assay.

Table 1. Primer sequences for PCR

Target gene	Primer	Sequence(5'-3')	Reference
eaeA	eaeA1	CAT TAT GGA ACG GCA GAG GT	15
	eaeA2	ATC TTC TGC GTA CTG CGT TCA	
eaeB	eaeB1	TAT CGA TAA TAA CAA TGC GG	15
	eaeB2	CAT GCG ATT AAT AAG GTC AG	
bfpA	bfpA1	AAT GGT GCT TGC GCT TGC TGC	16
	bfpA2	GCC GCT TTA TCC AAC CTG GTA	
EAF	EAF1	CAG GGT AAA AGA AAG ATG ATA A	17
	EAF2	TAT GGG GAC CAT GTA TTA TCA	
stx	stx1	GAA CGA AAT AAT TTA TAT GT	18
	stx2	TTT GAT TGT TAC AGT CAT	
fliC	fliC1	CAA GTC ATT AAT ACA AAC AGC C	19
	fliC2	GAC ATA TTG GAC ACT TCG GT	

Table 2. Characteristics of 54 *Escherichia coli* O111 Serotypes isolated from children with and without diarrheal

Serotype	No. of strains	clinical status	*eae*A	eaeB	*bfpA*	*EAF*	*stx*	HeLa adherence	
								3h	6h
O111:H34	1	D	+	-	+	+	-	LA	ND
O111:H12	1	D	+	+	+	-	-	LA	ND
O111:H9	1	D	+	+	+	-	-	LA	ND
O111:H14	1	D	+	-	+	-	-	LA	ND
O111:H3	1	D	+	+	+	-	-	LA	ND
O111:H8	1	D	+	-	-	-	-	NA	LLA
O111:H24	1	D	+	-	-	-	-	NA	LLA
O111:H9	1	D	+	+	-	-	-	NA	LLA
O111:H9	1	D	+	-	-	-	-	NA	LLA
O111:H21	2	D	+	+	-	-	-	NA	LLA
O111:H21	2	D	+	-	-	-	-	NA	LLA
O111:H46	1	D	+	-	-	-	-	NA	LLA
O111:H6	1	D	+	+	-	-	-	NA	LLA
O111:H12	1	D	+	-	-	-	-	NA	LLA
O111:H17	1	D	+	+	-	-	-	NA	LLA
O111:H20	1	D	-	-	+	-	-	NA	NA
O111:H21	1	D	-	-	-	-	+	NA	NA
O111:H34	1	D	-	-	-	-	+	NA	NA
O111	34	A, D	-	-	-	-	-	AA, DA	NA, UDP

eae: gene encoding intimin; bfpA: gene encoding bundle forming pilus; EAF: gene encoding EPEC adherence factor*; stx*: gene encoding Shiga toxin; D: diarrhea; A: asymptomatic; LA: Localized adherence; LLA: Localized Like adherence NA; nonadherence; UDP:undefined adherence; DA: difused adherence; AA: aggregative adherence; ND: not determined

The $\chi 2$ test, Fisher's exact test for 2×2 tables were used to verify differences between groups (statistical significance, $P< 0.05$).

Results

The *E. coli* serogroup O111 strains were isolated from 66.7% of the children with diarrhea versus 33.3% of healthy cases ($P< 0.05$). The O: H serotypes found in this study are listed in Table 2. Of the 54 *E.coli* serogroup O111 strains tested for the presence of flagella antigens, 47(89%) were typeable with standard H antisera and the remaining 7(11%) of the strains were non-motile (H -). Twenty-three different H type were distinguished among the O111 strains by PCR-RFLP analysis. The most common serotypes included: O111:H48 (16), O111:H21 (9), O111:H- (7), O111:H2 (5), O111:H9 (4), O111:H44 (3), O111:H10 (3); of which O111:H2, O111: H9, O111: H10, O111:H21 and O111:H4 serotypes. These were the most common in diarrheal cases ($P< 0.05$) (Table 2).

Table 3. Prevalence of virulence-related genes among *E. coli*: O111 strains isolated from children with diarrhea

Genetic profile	No (%) of patients
eaeA	7(35)
bfpA	1(5)
stx	2(10)
eaeA, eaeB	5(25)
eaeA, bfpA	1(5)
eaeA, eaeB, bfpA	3(15)
eaeA, bfpA, EAF	1(5)

Out of 54 strains that were studied, 34(70%) strains were negative for the presence of all virulence factors (*eaeA, eaeB, bfpA, EAF*, *stx* genes). Sixteen (47%) of 34 strains without any virulence genes were isolated from diarrheal cases and 18 (53%) from healthy individuals. Twenty (37%) strains isolated from diarrheal cases were positive for the presence of one or more of *eaeA, eaeB, bfpA, EAF* and *stx* virulence genes, of which 5 strains have localized adherence (LA), 12 strains localized like adherence (LLA) and 3 strains showed no adherence (NA) to HeLa cells (Table 4).

Out of 34(70%) negative strains for the presence of all virulence genes, 4, 3, 9 and 18 strains have DA (diffuse adherence), AA (aggregative adherence), UDP (undefined pattern) and NA (non adherence) adherence patterns on HeLa cells, respectively (Table 2). Localized adherence was found significantly more frequently in isolates from diarrheal cases (31.5%) than in those from the healthy controls ($P< 0.05$).

The *eaeA* gene was more prevalent in strains isolated from patients. The most profile of genotypes were $eaeA^{+}/stx^{-}$ (35%), $eaeA^{+}/eaeB^{+}/stx^{-}$(25%) as atypical EPEC:O111 followed by $eaeA^{+}/eaeB^{+}/bfpA^{+}/stx^{-}$ (15%) as typical EPEC:O111 and stx^{+} (10%) as STEC:O111. (table 3). A high percentage (22.2%) of atypical EPEC: O111 strains (having the *eaeA* gene but not *bfpA* gene) were isolated.

Table 4. Adhering and non-adhering *E. coli O111* isolated from diarrheal and healthy cases on HeLa cells

Adherence pattern	diarrhea (n=36)	healthy (n=18)
Aggregative adhesion	2	1
Diffuse adhesion	1	3
Localized adhesion	5	0
Localized Like adhesion	12	0
Not adherent	16	14

Discussion

Although the *E. coli* O111 serogroup is considered as a pathogenic strain, there is evidence that strains of this O group composed of strains with diverse H types, display a variety of virulence characteristics and mechanisms of pathogenesis. In our study, the most common serotypes detected in children with diarrhea were *E. coli* O111: H2, O111:H9, O111:H10, O111:H21 and O111:H4. Atypical EPEC: O111 serotypes ($eaeA^{+}$/ $bfpA^{-}$) were most frequently isolated from diarrheal cases. In similar studies, *E. coli* O111 serotypes have been dominant for more than two decades and are among the most important EPEC serotypes worldwide (22). We used PCR for characterizing the isolates according their virulence genes. PCR analysis revealed a significantly higher prevalence of the *eaeA gene* in strains isolated from patients than from the asymptomatic cases ($p<0.05$). Most EPEC strains have both bundle-forming pilus gene (*bfpA*) and *eaeA* gene, but in this study, most EPEC: O111 strains isolated were atypical EPEC (only contained the *eaeA* gene).

There were a limited number of H antigens associated with EHEC O111, including H21 and H34. The O111:H21 and O111:H34 serotypes have been implicated as a cause of enterohemorrhagic

diarrhea and were shown to be the source of the only reported EHEC O111 in this study.

The adherent *E. coli* strains in this study are similar to those in other reports (23-24) in that these are a cause of diarrhea (Table 3). Localized adhesion (LA), which is required for the full virulence of enteropathogenic *E. coli*, is conferred by bundle-forming pilus (*bfp)*, and is plasmid mediated (25, 26) by the enteropathogenic adherence factor (EAF). Our study showed that all *bfpA* positive strains manifest localized adhesion, confirming previous studies.

Our results are similar to those obtained by a study in that the aggregative adherent (AA) strains were isolated more frequently in symptomatic patients than in controls (27). There is controversy about the association between the diffuse adhesion (DA) and diarrhea. Some studies have reported a relationship (28-29), but the reverse association has also been reported (30). In this study, the diffuse adherent strains were isolated more frequently from healthy controls than from diarrheal cases. Robins-Browne *et al*. reported that there was no difference between patients and controls in the detection of *E. coli* with DA pattern (31). Mathewson *et al*. also detected DA strains as frequently in controls as in patients (32).

Our results showed a relationship between the combination of virulence genes and adherence patterns. The LA pattern of the *eaeA+/ bfpA+/ EAF+* strains may be explained by the presence of the EAF plasmid. We also demonstrated that the *fliC* RFLP approach is a reliable, rapid, and easy to perform method for determination of the H types of *E. coli* O111 clinical isolates, including nonmotile strains that cannot be H typed by serotyping.

In conclusion, this study emphasis the importance of epidemiological studies on *E. coli* O111 serotypes in our region. Application of multiple methodologies (such as serotyping, PCR, and adherence) that increase diagnostic sensitivity and specificity is strongly recommended. However, further investigations are needed for accurate identification and epidemiological study of these pathogens.

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