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Molecular Profiling and Phenotype Analysis of *Escherichia coli* O26:H11 and O26:NM: Secular and Geographic Consistency of Enterohemorrhagic and Enteropathogenic Isolates

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Fifty-eight enterohemorrhagic *Escherichia coli* O26:H11 or O26:NM (nonmotile) strains and 44 atypical enteropathogenic *E. coli* O26:H11 or O26:NM strains isolated from patients in 11 countries during 52 years share a common pool of non-*stx* virulence genes, fitness loci, and genotypic and phenotypic diagnostic markers. These findings indicate close relatedness between these pathotypes and provide a basis for their clinical laboratory diagnosis.

Escherichia coli strains expressing somatic (O) antigen 26 and flagellar (H) antigen 11 or no H antigen (i.e., they are nonmotile) (NM) are classified as either atypical enteropathogenic *E. coli* (aEPEC) (29) or enterohemorrhagic *E. coli* (EHEC) (18). aEPEC O26 strains, first identified as causes of infantile diarrhea in the 1940s (20), have caused pediatric diarrhea worldwide (11, 24, 29). Such strains contain *eae* encoding intimin (11), but in contrast to typical EPEC (20, 29), aEPEC O26 strains lack the EPEC adherence factor (EAF) plasmid (11, 24) encoding bundle-forming pili (20). They also lack genes encoding Shiga toxins (Stx), which are the major virulence factors of EHEC O26 (20). Since being recognized as pathogens >20 years ago (16, 17), EHEC O26 strains have emerged as the most common non-O157 EHEC serogroup causing hemolytic uremic syndrome (HUS) and diarrhea in Europe (5, 7, 28) and have been associated with human diseases worldwide (12, 14).

How related are EHEC O26 and aEPEC O26? Analysis of housekeeping genes (22) and comparative indexing of the core genomes (1) demonstrate that EHEC and aEPEC O26 share a variety of chromosomal regions. However, rosters of putative virulence characteristics of EHEC and aEPEC O26 have not been compared. Moreover, despite the importance of EHEC and aEPEC O26 as human pathogens, diagnostic procedures that identify both groups have not been established. To gain insight into their relatedness and to improve laboratory diagnosis of these organisms, we compared putative virulence genes of EHEC and aEPEC O26 isolates from patients from 11 countries, collected during more than 50 years, and sought genotypes and phenotypes that can be exploited to detect both of these pathogenic groups in clinical laboratories.

Bacterial strains. Fifty-eight EHEC and 44 aEPEC O26 strains were isolated between 1952 and 2003 from patients with HUS (25) (n = 31) or diarrhea (n = 71) in 11 countries (Austria [n = 2], Czech Republic [n = 22], Denmark [n = 3], France [n = 2], Germany [n = 59], Italy [n = 2], Norway [n = 1], the United Kingdom [n = 2], Australia [n = 1], Peru [n = 1], and the United States [n = 7]). Isolates from the same country showed no apparent geographical or temporal linkage. Sixty-two strains belonged to the serotype O26:H11, and 40 were of the serotype O26:NM. Restriction fragment length polymorphism analysis of the *fliC* gene demonstrated that all belong to the H11 clone complex.

Genotypic and phenotypic analyses. The strains were analyzed for putative virulence genes and diagnostic markers (Table 1) by PCRs as described previously (3, 4, 6, 7, 8, 9, 13, 21, 23, 26, 30). Long polar fimbriae of EHEC O26 (LPF_{O26}) (27) were identified with primers lpfAO26-1 (5′-CCGCTTGTGTT GTGTCTCC-3′) and lpfAO26-2 (5′-AGCAGATTTACCAG TATTCA-3′) derived from a sequence of the *lpfA* gene of *E. coli* O26:H11 strain 843/02 (27) (GenBank accession number AB161111); the PCR was performed in 30 cycles of denaturing (94°C, 30 s), annealing (50°C, 1 min), and extension (72°C, 1 min), followed by a final extension (72°C, 5 min). Molecular analysis of the high-pathogenicity island (HPI) was accomplished as previously described (15). The enterohemolytic phenotype was sought on enterohemolysin agar (2) (Sifin, Berlin, Germany). Tellurite resistance was determined based on an organism's ability to grow on cefixime-tellurite (CT)–sorbitol MacConkey agar (SMAC) (Oxoid, Hampshire, United Kingdom) and on CT-rhamnose MacConkey agar (CT-RMAC) (Sifin) (12). Urease activity was examined in urea degradation broth (Heipha, Heidelberg, Germany) after a 24-h incubation (37°C).

Stool analysis for *E. coli* O26. Two-hundred-microliter aliquots of enrichment cultures (7) from 636 stools from patients with HUS (n = 177) or diarrhea (n = 459) were inoculated on SMAC, CT-SMAC, and enterohemolysin agar between January 2002 and December 2003. The overnight cultures were PCR screened for stx_1, stx_2, *eae*, and rfb_{O157} (7). Seventy-eight rfb_{O157}-negative stools that were *stx* positive/*eae* positive or *stx*

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TABLE 1. Distribution of putative virulence genes and other loci among EHEC and aEPEC O26:H11/NM strains

Gene or plasmid	Predicted product or phenotype[a]	No. (%) of strains positive for the gene among:		P^b
		EHEC O26 (n = 58)	aEPEC O26 (n = 44)	
stx_1	Shiga toxin 1	30 (51.7)[c]	0	<0.0001[b]
stx_2	Shiga toxin 2	33 (56.9)[c]	0	<0.0001
EHEC-*hlyA*	EHEC hemolysin	47 (81.0)	33 (75.0)	0.80
cdt[d]	Cytolethal distending toxin	0	0	NA
eae β	Intimin β	58 (100.0)	44 (100.0)	1.00
efa-1[e]	EHEC factor for adherence (Efa1)	58 (100.0)	44 (100.0)	1.00
iha	IrgA homologue adhesin (Iha)	57 (98.3)	39 (88.6)	0.72
$lpfA_{O26}$	Major fimbrial subunit of LPF_{O26}	58 (100.0)	44 (100.0)	1.00
$lpfA_{O113}$	Major fimbrial subunit of LPF_{O113}	51 (87.9)	39 (88.6)	0.98
saa	STEC autoagglutinating adhesin (Saa)	0	0	NA
pEAF[f]	EAF plasmid	0	0	NA
bfpA	Bundle-forming-pili structural subunit	0	0	NA
irp-2	Iron-repressible protein 2	58 (100.0)	44 (100.0)	1.00
fyuA	Yersiniabactin receptor	58 (100.0)	44 (100.0)	1.00
ter[g]	Tellurite resistance	53 (91.4)	36 (81.8)	0.71
ure[h]	Urease	52 (89.7)	35 (79.5)	0.69

[a] IrgA, iron-regulated gene A; LPF, long polar fimbriae of EHEC O26 (LPF_{O26}) or EHEC O113 (LPF_{O113}); STEC, Shiga toxin-producing *E. coli*.
[b] The χ^2 test (a P value of <0.05 was considered significant). NA, not applicable.
[c] Five strains harbored both stx_1 and stx_2.
[d] *cdt-I, cdt-II, cdt-III, cdt-IV*, and *cdt-V* alleles (3) were investigated.
[e] The complete *efa-1* gene (ca. 10 kb) was identified in all strains.
[f] A 1-kb region of the EAF plasmid used as the EAF probe (pEAF) (6, 20) was targeted.
[g] All seven genes of the *ter* cluster of *E. coli* O157:H7 strain EDL933 (*terZ, terA, terB, terC, terD, terE*, and *terF*) (26) were present in ter^+ strains.
[h] All seven genes of the *ure* cluster of strain EDL933 (*ureD, ureA, ureB, ureC, ureE, ureF*, and *ureG*) (9) were present in ure^+ strains.

negative/*eae* positive were further investigated for *E. coli* O26 (described below).

Distribution of putative virulence and fitness genes among EHEC and aEPEC O26:H11/NM strains. The presence of *stx* genes in 58 EHEC strains and their absence in the 44 aEPEC strains was the only statistically significant difference (Table 1). All other genes investigated that encode various toxins and adhesins were either present with comparable frequencies among EHEC and aEPEC O26:H11/NM strains or absent from all strains of each pathotype (Table 1). The presence of *irp-2* and *fyuA*, components of the HPI which encodes an iron uptake system and has been proposed to be a "fitness island" (15), in each of the EHEC and aEPEC O26 strains (Table 1) prompted us to investigate whether a complete HPI is present in such strains. Two EHEC strains and two aEPEC strains were subjected to 14 additional PCRs which target the other HPI genes or link consecutive genes (15). This analysis demonstrated that each of the EHEC and aEPEC O26 strains contained a complete HPI which was structurally conserved; this further supports the close relatedness between these two pathotypes. Most EHEC and aEPEC O26 strains had genes encoding tellurite resistance (*terZ*, *terA*, *terB*, *terC*, *terD*, *terE*, and *terF*) (26) and urease production (*ureD*, *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, and *ureG*) (9) (Table 1). The finding of the widespread distribution of the *ure* genes within both *E. coli* O26 pathotypes is particularly important in light of a recent work (19) which demonstrated that the presence of the *ureC* gene distinguishes EHEC of the major serogroups, including O26, from diarrheagenic *E. coli* of other pathotypes, including EPEC; based on this, *ureC* was recommended as a target to screen for EHEC (19). In contrast, our data demonstrate that *ureC* is also a suitable target to seek aEPEC O26. However, in accordance with the observation by Nakano et al. (19), urease activity was expressed by only a minority of ure^+ EHEC and aEPEC O26 strains (Table 2), indicating that the urease phenotype is not a suitable diagnostic marker for these strains.

Diagnostically useful phenotypes of EHEC and aEPEC O26:H11/NM strains. Each of 47 EHEC O26:H11/NM strains and 33 aEPEC O26:H11/NM strains that harbored the EHEC *hlyA* gene demonstrated an enterohemolytic phenotype (Table 2).

TABLE 2. Genotypic and phenotypic characteristics of EHEC and aEPEC O26:H11/NM strains with potential diagnostic utility

Group	Total no. of strains	No. of strains with gene(s)/phenotype[a]			No. (%) of strains with phenotype combination[f]			
		EHEC *hlyA*/ EHEC-Hly[b]	*ter*/CT-SMAC/ CT-RMAC[c]	*ure* genes[d]/ urease activity[e]	EHEC-Hly$^+$ CT-media$^+$	EHEC-Hly$^-$ CT-media$^+$	EHEC-Hly$^+$ CT-media$^-$	EHEC-Hly$^-$ CT-media$^-$
EHEC O26	58	47/47	53/53/53	52/1	43 (74.1)	10 (17.3)	4 (6.9)	1 (1.7)
aEPEC O26	44	33/33	36/36/36	35/2	28 (63.6)	8 (18.2)	5 (11.4)	3 (6.8)

[a] The remaining strains of each group were negative for the genes and the corresponding phenotypes.
[b] EHEC-Hly, enterohemolytic phenotype.
[c] Presence of *ter* genes (*terZ, terA, terB, terC, terD, terE*, and *terF*)/growth on CT-SMAC/growth on CT-RMAC. The growths on CT-SMAC and CT-RMAC were comparable with those on the corresponding media without CT.
[d] All *ure* genes (*ureD, ureA, ureB, ureC, ureE, ureF*, and *ureG*) were present.
[e] As detected in the urea degradation broth after a 24-h incubation.
[f] CT-media, growth on CT-SMAC and CT-RMAC; +, the phenotype was present; −, the phenotype was absent.

TABLE 3. Isolation of EHEC and aEPEC O26 from stool samples by colony blot hybridization, culture on enterohemolysin agar, and culture on CT-SMACs

Result of stool PCR screening	No. of stools with PCR result	No. of *E. coli* O26 strains isolated by:				
		Colony blot hybridization with probe:			Entero-hemolysin agar	CT-SMAC
		stx$_1$	*stx*$_2$	*eae*		
stx$^+$/*eae*$^+$	48	6[a]	8[a]	14[a]	13[b]	10[c]
stx negative/*eae*$^+$	30	0	0	5	4[b]	3[c]
Total	78	6	8	19	17[d]	13[e]

[a] The colonies which hybridized with the *stx*$_1$ or *stx*$_2$ probe also hybridized with the *eae* probe.
[b] One EHEC strain and one aEPEC strain that were missed on enterohemolysin agar were identified on CT-SMAC.
[c] Four EHEC strains and two aEPEC strains that were missed on CT-SMAC were identified on enterohemolysin agar.
[d] All strains contained EHEC *hlyA*; this gene was absent from the two strains that could not be detected on enterohemolysin agar.
[e] All strains contained *ter* genes; these genes were also present in the six strains that could not be isolated on CT-SMAC because of their low numbers in stools.

Moreover, the presence of *ter* genes was perfectly correlated with tellurite resistance of EHEC and aEPEC O26 strains (Table 2). On CT-SMAC, all strains fermented sorbitol. In contrast, on CT-RMAC, 50 (94.3%) of 53 EHEC O26 strains and 30 (83.3%) of 36 aEPEC O26 strains generated rhamnose-nonfermenting, colorless colonies, allowing their differentiation from rhamnose-fermenting (reference 12 and this study), normal intestinal flora. Most EHEC and aEPEC O26 strains displayed both enterohemolytic and tellurite resistance phenotypes (Table 2). Only four of the 102 *E. coli* O26 strains investigated displayed neither of these two phenotypes (Table 2), because they lacked the encoding genes and could not be, therefore, identified on either enterohemolysin agar or tellurite-containing media.

Isolation of EHEC and aEPEC O26 from stools. To validate the utility of the enterohemolytic and tellurite resistance phenotypes for isolating EHEC and aEPEC O26 from stools, 78 fecal samples that were *stx* positive/*eae* positive or *stx* negative/*eae* positive by PCR screening were subjected to colony blot hybridization with *stx*$_1$, *stx*$_2$, and *eae* digoxigenin-labeled probes (7) (used as a gold standard). In parallel, they were cultured on enterohemolysin and CT-SMACs. Resulting enterohemolytic and sorbitol-fermenting colonies, and hybridizing colonies from master plates, were screened by slide agglutination with O26 antiserum. By use of colony blot hybridization, 14 EHEC O26 strains and 5 aEPEC O26 strains were isolated (Table 3). Enterohemolysin agar culture identified 13 of the 14 EHEC strains and 4 of the 5 aEPEC strains detected by colony blot hybridization (sensitivities of 92.9% and 80.0%, respectively) (Table 3). Culture on CT-SMAC identified 10 of the 14 EHEC strains and 3 of the 5 aEPEC strains identified by colony blot hybridization (sensitivities of 71.4% and 60.0%, respectively) (Table 3). The two strains that were not detected on enterohemolysin agar lacked the EHEC *hlyA* gene. In contrast, the six strains which were missed on CT-SMAC contained complete *ter* operons. The failure to identify these strains probably resulted from low numbers of these organisms in the respective stools, as indicated by colony blot hybridization, combined with the absence of a distinct sorbitol fermentation phenotype. CT-SMAC is a superior medium for the isolation of non-sorbitol-fermenting EHEC O157:H7 (7, 20); it was also successfully used to isolate EHEC O26 from acid-pretreated bovine feces (10). However, it does not distinguish sorbitol-fermenting *E. coli* O26 from other intestinal coliforms that are usually, but not always, reduced in density on CT-SMAC, because some of them also contain *ter* genes (26). This might explain why the sensitivity of CT-SMAC for the isolation of *E. coli* O26 in our study was lower than that of enterohemolysin agar, which allows a direct phenotypic differentiation of these pathogens from normal intestinal flora. However, culture on CT-SMAC identified the two EHEC *hlyA*-negative strains that were not detected on enterohemolysin agar. Conversely, all six strains that were missed on CT-SMAC were detected on enterohemolysin agar. Taken together, the results of this 2-year prospective study demonstrated that the combined culture on enterohemolysin agar and CT-SMAC is a simple and sensitive procedure to isolate both EHEC and aEPEC O26 from *eae*-positive stool samples.

Our data demonstrate that EHEC and aEPEC O26:H11/NM strains are largely conserved, without evidence of geographic or secular variation. These two pathotypes share a common pool of fitness and virulence genes, with *stx* genes being the only difference identified. Some of the easily detectable phenotypes shared by EHEC and aEPEC O26 strains, such as an enterohemolytic phenotype and growth on tellurite-containing media, can be exploited for the isolation of these pathogens from stool samples.

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