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Low-Density Macroarray Targeting Non-Locus of Enterocyte Effacement Effectors (*nle* Genes) and Major Virulence Factors of Shiga Toxin-Producing *Escherichia coli* (STEC): a New Approach for Molecular Risk Assessment of STEC Isolates▽

Marie Bugarel,[1] Lothar Beutin,[2] and Patrick Fach[1]*

AFSSA (French Food Safety Agency), Food Quality and Agro-alimentary Processes Research Laboratory (LERQAP), 23 Avenue du Général De Gaulle, F-94706 Maisons-Alfort, France,[1] and National Reference Laboratory for Escherichia coli (NRL-E. coli), Federal Institute for Risk Assessment (BfR), Diedersdorfer Weg 1, D-12277 Berlin, Germany[2]

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Rapid and specific detection of Shiga toxin-producing *Escherichia coli* (STEC) strains with a high level of virulence for humans has become a priority for public health authorities. This study reports on the development of a low-density macroarray for simultaneously testing the genes *stx*$_1$, *stx*$_2$, *eae*, and *ehxA* and six different *nle* genes issued from genomic islands OI-122 (*ent*, *nleB*, and *nleE*) and OI-71 (*nleF*, *nleH1-2*, and *nleA*). Various strains of *E. coli* isolated from the environment, food, animals, and healthy children have been compared with clinical isolates of various seropathotypes. The *eae* gene was detected in all enteropathogenic *E. coli* (EPEC) strains as well as in enterohemorrhagic *E. coli* (EHEC) strains, except in EHEC O91:H21 and EHEC O113:H21. The gene *ehxA* was more prevalent in EHEC (90%) than in STEC (42.66%) strains, in which it was unequally distributed. The *nle* genes were detected only in some EPEC and EHEC strains but with various distributions, showing that *nle* genes are strain and/or serotype specific, probably reflecting adaptation of the strains to different hosts or environmental niches. One characteristic *nle* gene distribution in EHEC O157:[H7], O111:[H8], O26:[H11], O103:H25, O118:[H16], O121:[H19], O5:H−, O55:H7, O123:H11, O172:H25, and O165:H25 was *ent*/*espL2*, *nleB*, *nleE*, *nleF*, *nleH1-2*, *nleA*. (Brackets indicate genotyping of the *flic* or *rfb* genes.) A second *nle* pattern (*ent*/*espL2*, *nleB*, *nleE*, *nleH1-2*) was characteristic of EHEC O103:H2, O145:[H28], O45:H2, and O15:H2. The presence of *eae*, *ent*/*espL2*, *nleB*, *nleE*, and *nleH1-2* genes is a clear signature of STEC strains with high virulence for humans.

Since the early 1980s, Shiga toxin-producing *Escherichia coli* (STEC) has emerged as a major cause of food-borne infections (17, 30). STEC can cause diarrhea in humans, and some STEC strains may cause life-threatening diseases, such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). On the basis of its human pathogenicity, this subset of STEC strains was also designated enterohemorrhagic *E. coli* (EHEC) (22, 25). Numerous cases of HC and HUS have been attributed to EHEC serotype O157:H7 strains, but it has now been recognized that other serotypes of STEC belong to the EHEC group. The STEC seropathotype classification is based upon the serotype association with human epidemics, HUS, and diarrhea and has been developed as a tool to assess the clinical and public health risks associated with non-O157 EHEC and STEC strains (18). Only a few serotypes of STEC have been reported as most frequently associated with severe disease in humans. Besides *E. coli* O157:[H7], five other serotypes, namely O26:[H11], O103:H2, O111:[H8], O121:[H19], and O145:[H28], account for the group of typical EHEC (25). (Brackets indicate genotyping of the *flic* or *rfb* genes; the absence of brackets indicates data obtained with the conventional serotyping approach using specific antisera, as described in Materials and Methods.) Atypical EHEC group strains of serotypes O91:[H21], O113:H21, and O104:H21 are less frequently involved in hemorrhagic diseases than typical EHEC but are a frequent cause of diarrhea (8, 12, 25). Recent data from Enter-Net, a global surveillance consortium of 35 countries that tracks enteric infectious diseases, showed that the number of human cases of illness caused by non-O157 EHEC increased globally by 60.5% between 2000 and 2005, while at the same time the number of cases linked to EHEC O157 increased by only 13% (1). In the past few years, new serotypes of EHEC that differ from those previously known as typical and atypical EHEC have emerged (6, 8, 23, 24, 31). These EHEC strains were identified as important causes of food-borne infections in humans and were described as "new emerging EHEC."

The production of Shiga toxin (Stx) by EHEC is the primary virulence trait responsible for HUS, but many *E. coli* non-O157:H7 strains that produce Stx do not cause HUS. Identification of human-virulent STEC by detection of unique *stx* genes may be misleading, since not all STEC strains are clinically significant for humans (11). Besides the ability to produce one or more types of Shiga toxins, typical EHEC strains harbor a genomic island called the "locus of enterocyte effacement" (LEE). Atypical EHEC strains are negative for the LEE but may carry other factors for colonization of the human

* Corresponding author. Mailing address: Agence Française de Sécurité Sanitaire des Aliments (Afssa), 23, avenue du Général De Gaulle, F-94706 Maisons-Alfort, France. Phone: 33 (0) 14977 2813. Fax: 33 (0) 14568 9762. E-mail: p.fach@afssa.fr.

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intestine (6, 25). The LEE carries genes encoding functions for bacterial colonization of the gut and for destruction of the intestinal mucosa, thus contributing to the disease process (25). The LEE *eae* gene product intimin is directly involved in the attaching and effacing (A/E) process (37). The LEE includes regulatory elements, a type III secretion system (TTSS), secreted effector proteins, and their cognate chaperon (13, 29). In addition to the intimin, most of the typical EHEC strains harbor the plasmid-borne enterohemolysin (*ehxA*), which is considered an associated virulence factor (6, 25).

A number of other pathogenicity island (PAI) candidates, including O island 122 (OI-122) and O island 71 (OI-71), have been found in EHEC and EPEC strains, but their role in disease is not fully clear. Within the EHEC group, both O157:H7 strains (19, 34) and non-O157 strains (18, 35) present a variable repertoire of virulence determinants, including a collection of non-LEE-encoded effector (*nle*) genes that encode translocated substrates of the type III secretion system (9, 20). Our objective was to identify type III secreted virulence factors that distinguish EHEC O157 and non-O157 strains constituting a severe risk for human health from STEC strains that are not associated with severe and epidemic disease, a concept called "molecular risk assessment" (MRA) by Coombes et al. (9). Supporting the MRA approach requires the development of diagnostic tests based on multiplex nucleic acid amplification and microfluidics-based detection using standardized platforms applicable in hospital service or public health laboratories. It is now feasible to develop low-density DNA arrays that can be used to examine the gene inventory from isolated strains, offering a genetic bar coding strategy. A recent innovation in this field is the introduction of the GeneSystems PCR technology (5, 36). In this study, we have developed a GeneDisc array designed for simultaneous detection of genes encoding Shiga toxins 1 and 2 (stx_1 and stx_2), intimins (*eae*), enterohemolysin (*ehxA*), and six different *nle* genes derived from genomic islands OI-71 and OI-122. We focused our efforts on the detection of the OI-122 genes, *ent*/*espL2* (*Z4326*), *nleB* (*Z4328*), and *nleE* (*Z4329*), and the OI-71 genes, *nleF* (*Z6020*), *nleH1-2* (*Z6021*), and *nleA* (*Z6024*). The macroarray presented here was evaluated for its specificity and ability to discriminate between STEC causing serious illness in humans and other *E. coli* strains.

MATERIALS AND METHODS

Principle of the GeneDisc array. The principle of the GeneDisc array (Gene Systems, Bruz, France) has been previously described (5). It is based on real-time PCR applications of multiple targets in a plastic reaction tray engraved with reaction microchambers preloaded with desiccated PCR primers and TaqMan probes labeled with either the reporter dye 6-carboxyfluorescein (6-FAM; 490 to 520 nm) or carboxy-X-rhodamine (ROX; 580 to 620 nm).

Properties of the GeneDisc array developed in this study. The "virulotyping GeneDisc" was designed for simultaneous examination of six different samples, each being tested for 10 EHEC-specific gene targets, together with negative and inhibition controls. It has the following settings: microwell 1, negative PCR control (6-FAM label) and PCR inhibition control (ROX-label); microwell 2, stx_2 (FAM) and stx_1 (ROX); microwell 3, *ent*/*espL2* (FAM) and *nleF* (ROX); microwell 4, *nleB* (FAM) and *nleH1-2* (ROX); microwell 5, *nleE* (FAM) and *nleA* (ROX); and microwell 6, *ehxA* (FAM) and *eae* (ROX). The oligonucleotide primers and gene probes used in the GeneDisc are listed in Table 1. Primers and probes used for detecting stx_1, stx_2, and *eae* were described previously (26, 28) and were evaluated for their specificity and sensitivity (5). All oligonucleotides were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). GeneDisc spotting and manufacturing were performed by GeneSystems (Bruz, France).

Bacterial strains investigated with the GeneDisc array. Strains of *E. coli* and other *Enterobacteriaceae* that were investigated for their virulence gene content with the virulotyping GeneDisc were from the collection of the National Reference Laboratory for *E. coli* (NRL-E. coli) at the Federal Institute for Risk Assessment (BfR) in Berlin, Germany, and from the French Food Safety Agency (AFSSA) in Maisons-Alfort, France. We used *eae*-positive attaching and effacing *E. coli* strains that were characterized by their *eae* alleles as previously described (21). STEC strains used in this study have been characterized by their *stx* genotypes as previously reported (2, 4, 21). All strains investigated in this work were identified for the *E. coli* O (lipopolysaccharide) and H (flagellar) antigens with specific antisera produced at the NRL-E. coli at the BfR as previously described (21). For reference strains of EHEC O groups O26, O103, O111, O145, and O157, we used strains previously identified by serotyping of their O and H antigens and by *fliC* genotyping (3). The characteristics and origin of EHEC reference strains H19 (O26:H11), PMK5 (O103:H2), CL37 (O111:[H8]), CB7874 (O145:[H28]), and EDL933 (O157:H7) had been described in other publications (3, 27, 32). The EHEC strain EDL933 (O157:H7) and the EPEC strain E2348/69 (O127:H6) were used as positive controls for testing the complete set of *nle* genes, i.e., *ent*/*espL2* (*Z4326*), *nleB* (*Z4328*), *nleE* (*Z4329*), *nleF* (*Z6020*), *nleH1-2* (*Z6021*), and *nleA* (*Z6024*). Strain C600 (*E. coli* K-12) was taken as a negative control for all genes investigated in this work (4). In addition, 97 enterobacteriaceal strains (from *Cronobacter sakazakii*, *Yersinia* spp., *Escherichia* spp., *Salmonella* spp., *Shigella* spp., *Citrobacter* spp., *Hafnia* spp., *Klebsiella* spp., and *Proteus* spp.) that were characterized by standard methods (14) were used for evaluation of the GeneDisc array. Except for *Shigella dysenteriae* type 1 (stx_1), the *Shigella sonnei* strain CB7888 (stx_1) (4), and the *Citrobacter rodentium* strain 10835 (*eae*), all other *Enterobacteriacae* isolates were negative for *stx* and *eae* genes. For examination, bacteria were cultured to single colonies on Luria broth plates and grown overnight at 37°C. A small aliquot of the colony corresponding to approximately 2×10^6 bacteria was either DNA extracted using the InstaGene matrix (Bio-Rad Laboratories, Marnes La Coquette, France) or directly added to 200 μl sterile water and vortexed thoroughly. Thirty-six microliters of the resuspended bacteria or DNA extracts was tested by the GeneDisc array.

RESULTS

Association of *eae* types, *ehxA* gene, and *nle* genes with typical and atypical EHEC strains. 243 EHEC strains, including typical EHEC ($n = 183$), atypical EHEC ($n = 18$), and new emerging EHEC strains ($n = 41$) as well as *stx*-negative strains belonging to the same serotype as the EHEC strains ($n = 65$), were investigated with the virulotyping GeneDisc array (Tables 2, 3, and 4). All EHEC strains tested positive for stx_1 and/or stx_2 genes, giving a total concordance with data previously published (3, 5, 15, 28). *eae* genes were detected in the strains belonging to the classical EHEC groups O26, O103, O111, O121, O145, and O157 as well as in emerging EHEC type O5, O15, O45, O55, O118, O123, O165, and O172 strains. Only one EHEC O103:H2 strain tested negative for the *eae* genes (Table 2). *eae* genes were absent in all other STEC strains investigated, including atypical EHEC O91:H21 and O113:H21; these strains are frequently isolated from food and from human patients (33). All *eae*-negative STEC strains as well as the atypical EHEC strains were also negative for the set of *nle* genes investigated in this study (Table 4). *nle* genes borne by islands OI-71 and OI-122 were present in typical EHEC strains, including the new emerging serotypes. One characteristic pattern of *nle* genes (*ent*/*espL2*, *nleB*, *nleE*, *nleF*, *nleH1-2*, and *nleA*) was found in EHEC strains belonging to serotypes O157:[H7], O111:[H8], O26:[H11], O103:H25, O118:[H16], O121:[H19], O5:H−, O55:H7, O123:H11, O172:H25, and O165:H25 (Table 2). Among the 76 EHEC O157:[H7] strains, six were sorbitol-fermenting (SF) O157:H_{NM} stx_2 strains; these showed the same *nle* pattern as the non-SF O157:[H7] strains. Two O-rough:[H7] (stx_2, *eae*-gamma) strains, previously iden-

TABLE 1. Primers and probes preloaded in the GeneDisc

Target gene sequence[a]	Forward primer, reverse primer, and probe sequences (5′–3′)[b]	Location within sequence	GenBank accession no.	Reference
stx_1	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	878–906	M16625	28
	CCC CAG TTC ARW GTR AGR TCM ACR TC*	983–1008		
	ROX- CTG GAT GAT CTC AGT GGG CGT TCT TAT GTA A-BHQ	941–971		
stx_2	TTT GTY ACT GTS ACA GCW GAA GCY TTA CG	785–813	X07865	28
	CCC CAG TTC ARW GTR AGR TCM ACR TC*	887–912		
	FAM- TCG TCA GGC ACT GTC TGA AAC TGC TCC-BHQ	838–864		
eae	CAT TGA TCA GGA TTT TTC TGG TGA TA	899–924	Z11541	26
	CTC ATG CGG AAA TAG CCG TTA*	979–1000		
	ROX- AT AGT CTC GCC AGT ATT CGC CAC CAA TAC-BHQ*	936–966		
ehxA	GTG TCA GTA GGG AAG CGA ACA	41832–41852	AF074613	This study
	ATC ATG TTT TCC GCC AAT G*	41939–41957		
	FAM- CGT GAT TTT GAA TTC AGA ACC GGT GG-BHQ	41868–41893		
ent/espL2	TCC TGG ATT ATT TTC TGC ATT TCA	3929758–3929781	AE005174	This study
	ACT ATT GCC AAG TAC GCC ACA A*	3929833–3929812		
	FAM-AAT GGT CAT GCA GAC GCA ATA AAG GCA TA-BHQ	3929783–3929811		
nleB	CAT GTT GAA GGC TGG AAS TTT GT	3931502–3931524	AE005174	This study
	CCG CTA CAG GGC GAT ATG TT*	3931573–3931554		
	FAM-ACA GAG ACG GGA AAA ACT GGA TGC CA-BHQ	3931527–3931552		
nleE	AGA AGC GTT TGA ACC TAT TTC CA	3932207–3932229	AE005174	This study
	TTG GGC GTT TTC CGG ATA T*	3932289–3932271		
	FAM-AGC CAG TAC ACC GGA AGG AAG CTG G-BHQ	3932237–3932261		
nleF	TGA GGT GAG AAA TGA AAA TAC TGA TG*	2281256–2281231	AE005174	This study
	CTA TCC CTG TCC TCT ATC GTC ATTC	2281182–2281206		
	ROX-TGT CGG AGC GCT GAG GGC G-BHQ*	2281226–2281208		
nleH1-2	ACA AGA GAA AGT CAT AGT GGT TG	2282298–2282276	AE005174	This study
	AAT CTC YCC CTT AGG CCA TCC CA*	2282230–2282252		
	ROX-TTT ACT AAT CTG TTG CAC AGG-BHQ	2282274–2282254		
nleA	AGA TAA CYC TAA TAC TAA ATA TGC C	2285138–2285162	AE005174	This study
	GCC CAA CCA TTG CRC CGA TAT GAG G*	2285274–2285250		
	ROX-TTC TTA CCA ATG CTG CCG CAA ATG CGC-BHQ	2285164–2285190		

[a] stx_1, gene encoding Shiga toxin 1; stx_2, gene encoding Shiga toxin 2; *eae*, gene encoding intimin; *ehxA*, gene encoding enterohemolysin; *ent/espL2*, gene encoding the putative non-LEE effector ent/espL2; *nleB*, gene encoding the putative non-LEE effector B; *nleE*, gene encoding the putative non-LEE effector E; *nleF*, gene encoding the putative non-LEE effector F; *nleH1-2*, gene encoding the putative non-LEE effector H1-2; *nleA*, gene encoding the putative non-LEE effector A.

[b] In the sequence of oligonucleotides: Y, (C, T); S, (C, G); W, (A, T); R, (A, G); M, (A, C); K, (G, T); H, (A, T, C); D, (G, A, T). FAM, 6-carboxylfluorescein; ROX, carboxy-X-rhodamine; BHQ, black hole quencher; *, complementary strand.

tified as positive for the $rfbE_{O157}$ gene, had the same *nle* pattern as serologically typeable O157:[H7] strains.

Another type of *nle* pattern was found with EHEC strains belonging to serotypes O103:H2, O145:[H28], O45:H2, and O15:H2. These were positive for all *nle* genes investigated except for OI-71-borne genes *nleA* and *nleF* (Table 2). Our results indicate that typical EHEC strains are highly conserved for the distribution of *nle* genes and point to an association of *eae* genotype, *nle* pattern, and serotype. Exceptions were rarely observed, such as an absence of the *nleH1-2* gene in one of the 34 examined EHEC O26:H11 strains (Table 2). Most (93.25%) of the typical EHEC strains were positive for the plasmid-located *ehxA* gene encoding enterohemolysin; this marker was also present in 87% of new emerging EHEC, in 73% of the atypical EHEC, and in 42.66% of the other STEC strains investigated in this study.

Identification and characterization of *stx*-negative strains resembling EHEC for serotype and other properties. It was previously reported that EHEC strains can lose their *stx* genes spontaneously during infection and upon subculturing (16). We were interested in investigating *stx*-negative, *eae*-positive *E. coli* strains belonging to EHEC-associated serotypes for their similarity with EHEC strains in regard to their *eae* genotypes and their *nle* genes. The results obtained with 65 strains are presented in Table 3. We could identify three *stx*-negative O157:[H7], 10 O26:[H11], one O103:[H2], three O121:[H19], one O121:H−, and one O15:H2 strain that showed *eae* genotypes and *nle* patterns similar to those exhibited by Stx-producing EHEC strains belonging to the same serotypes (Table 3). It seems likely that these strains represent variants of EHEC strains belonging to these serotypes that have lost their *stx* genes. In contrast, *stx*-negative strains belonging to the same O groups but showing H types other than those of the classical EHEC strains differed also with respect to their *eae* genotypes and the presence and types of *nle* genes (Table 3), indicating that these were not variants of classical EHEC

TABLE 2. Virulotyping of the *eae* and *nle* genes in EHEC strains

Serotype	No. tested	*stx* pattern	Virulotype							
			ehxA	*eae*	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
Or[157]:H7, O157:[H7][a]	68[b,c,d]	3 stx_1, 43 stx_2, 22 stx_1 and stx_2	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O157:[H7]	6[b,c,d]	1 stx_1, 4 stx_2, 1 stx_1 and stx_2	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O157:[H7]	2[b]	2 stx_2	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O103:H2	23[b,c,d]	21 stx_1, 1 stx_2, 1 stx_1 and stx_2	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O103:H2	2[b,d]	2 stx^1		epsilon	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O103:[H2]	1	stx_1	*ehxA*		*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O111:[H8]	21[b,d]	13 stx_1, 3 stx_2, 5 stx_1 and stx_2	*ehxA*	theta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O111:[H−]	2[b]	1 stx_2, 1 stx_1 and stx_2	*ehxA*	theta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O111:H8	1[b]	stx_1		theta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O111:H8	1[b]	stx_1 and stx_2		theta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O26:[H11]	22[b,c,d]	12 stx_1, 7 stx_2, 3 stx_1 and stx_2	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O26:[H11]	7[b]	2 stx_1, 3 stx_2, 2 stx_1 and stx_2		beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O26:[H11]	3[b,d]	2 stx_1, 1 stx_1 and stx_2	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O26:[H11]	1[b]	stx_1		beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O26:H11	1[b]	stx_1	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*			*nleA*
O145:[H28]	17[b,d]	8 stx_1, 8 stx_2, 1 stx_1 and stx_2	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O145:H28	1[b]	stx_1	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O121:H19	4[b]	4 stx_2	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O5:H−	12[b,c]	12 stx_1	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O103:H25	1[b]	stx_2	*ehxA*	theta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O55:H7	2[b,c]	2 stx_2		gamma	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O118:H16	19[b,d]	16 stx_1, 3 stx_1 and stx_2	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O118:H16	2[b]	2 stx_1		beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O123:H11	1[b]	stx_2	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O165:H25	1[b]	stx_2	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O172:[H25]	1[b]	stx_2	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O45:H2	1[b]	stx_1	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O15:H2	1[b]	stx_2		beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	

[a] Six of these were SF O157:H− and two were O-rough:[H7] positive for the $rfbE_{O157}$ gene. Brackets indicate genotyping of the *flic* or *rfb* genes.
[b] Clinical isolate.
[c] Food isolate.
[d] Animal/environment isolate.

strains. EHEC O111:[H8] strains were usually positive for *eae*-theta and for all OI-71- and OI-122-encoded *nle* genes. Only one of 25 strains was negative for *nleF* (Table 2). Three *stx*-negative strains (O111:H11 and O111:[H25]) showed *nle* genotypes similar to those of EHEC O111:[H8]. The differences in the H type and in the *eae* genotype indicate that these were not closely related to strains of the EHEC O111:[H8] group. All other O111 strains, including EPEC O111:H2, were different from EHEC O111:[H8] with respect to their *nle* genotypes (Table 3).

EHEC O145:[H28] strains are characterized by possession of the complete set of OI-122 module 2-associated *nle* genes *ent*, *nleB*, and *nleE* (Table 2). Interestingly, these genes were absent in two Stx-negative O145:[H28] strains which resemble O145:[H28] EHEC with respect to all other traits that were investigated (Table 3). It cannot be excluded that these strains have lost their *stx* genes and the OI-122 PAI. All EPEC O145 strains differed significantly from EHEC O145:[H28] as they do not possess any *nle* gene and carry other *eae* genotypes.

In the group of O103:H2 strains, the rabbit EPEC strain E22 was similar to all EHEC O103:H2 strains for the set of *nle* genes but differed in the *eae*-beta subtype, as EHEC O103:H2 strains carry *eae*-epsilon. In contrast, the EHEC O103:H25 strain which caused an outbreak of HUS in Norway in 2006 (31) was found to be different from the classical EHEC O103:H2 clone by its H type, its *eae* type, and the set of *nle* genes.

We additionally investigated representatives of classical EPEC groups. The EPEC O55:H7 strain was similar in its *eae* genotype and *nle* genes to EHEC O157:[H7] and EHEC O55:H7 strains. All *nle* genes investigated were also present in the EPEC O127:H6 reference strain E2348/69. EPEC O84:H2 harbored all *nle* genes except *nleE*. EPEC O156:H8 was negative only for the OI-71 *nleF* and *nleA* genes. EPEC O128:H2 and O113:H6 were positive only for *nleH* and lacked the OI-122 module 2-associated *nle* genes. EPEC O55:H6 also lacked the OI-122 module 2-associated *nle* genes but carried *nleH* and *nleF*. In contrast, EPEC O86:H40 carried the OI-122 module 2-associated *nle* genes but none of those located on OI-71 (Table 3). Some other EPEC strains (O125:H6, O126:H6, O51,

TABLE 3. Virulotyping of the *eae* and *nle* genes in *stx*-negative strains

Serotype	No. tested	Virulotype							
		ehxA	*eae*	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O103:H2	1	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O103:H2	1[a]		beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O111:H11	2	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O111:[H25]	1		theta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O111:[H2]	2		beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O111:NM	1		beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O111:H19	2		eta						
O145:[H28]	2	*ehxA*	gamma					*nleH1-2*	
O145:H34	1		theta						
O145:H4	1		iota						
O145:Hr	1		iota						
O26:[H11]	7		beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O26:H11	2	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O26:[H11]	1	*ehxA*	beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O26:[H11]	1		beta				*nleF*	*nleH1-2*	*nleA*
O26:[H11]	1						*nleF*	*nleH1-2*	*nleA*
O157:H7	1[b]	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O157:[H7]	2	*ehxA*	gamma	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	*nleA*
O157:[HNT]	1		beta					*nleH1-2*	*nleA*
O157:H16	5		epsilon					*nleH1-2*	*nleA*
O157:H2	1		tau					*nleH1-2*	*nleA*
O157:H26	2		beta					*nleH1-2*	*nleA*
O157:H27	1	*ehxA*	Nontypeable			*nleE*			*nleA*
O157:H39	1	*ehxA*	kappa			*nleE*			*nleA*
O157:H45	2		alpha				*nleF*	*nleH1-2*	*nleA*
O157:H45	1		alpha				*nleF*	*nleH1-2*	
O121:H19	3	*ehxA*	epsilon	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O121:H19	1	*ehxA*		*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O121:H−	1		epsilon	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O55:H7	4		gamma	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O15:H2	1		beta	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O127:H6	1[c]		alpha	*ent/espL2*	*nleB*	*nleE*	*nleF*	*nleH1-2*	*nleA*
O156:H8	1		gamma	*ent/espL2*	*nleB*	*nleE*		*nleH1-2*	
O84:H2	1	*ehxA*	zeta	*ent/espL2*	*nleB*		*nleF*	*nleH1-2*	*nleA*
O86:H40	1		theta	*ent/espL2*	*nleB*	*nleE*			
O55:H6	1		iota				*nleF*	*nleH1-2*	
O128:H2	1		beta					*nleH1-2*	
O113:H6	1		beta					*nleH1-2*	
O125:H6	1		alpha						
O126:H6	1		alpha						
O51	1		alpha						
O76:H51	1		gamma						

[a] Reference strain E22.
[b] Reference strain ATCC43888.
[c] Reference EPEC strain E2348/69.

and O76:H51) did not possess any *nle* genes and usually carried the *eae*-alpha genotype.

Identification and characterization of *eae*- and *nle*-negative strains. Numerous types of STEC are isolated from animals and food, but only 5% of these are positive for an *eae* gene or belong to serogroups O26, O103, O111, O145, and O157 (2). Some of the *eae*-negative STEC strains are known to cause diarrhea in humans but are rarely involved in hemorrhagic diseases such as HC and HUS (3, 16, 33). We were interested in investigating representative strains of the *eae*-negative STEC types that are frequently isolated from food (O8, O91, O100, O113, O146, O128, and O174). A total of 149 STEC strains that were isolated from food, animals, and humans as well as 29 fecal *E. coli* isolates from healthy children (FEC) were investigated with the virulotyping GeneDisc. The results show that the *eae*-negative STEC strains also tested negative for the *nle* genes (Table 4). All the *E. coli* strains issued from fecal flora of healthy infants were also found negative for *stx*, *eae*, and the *nle* genes. These data corroborate previous findings (18) indicating the absence of the LEE as well as the OI-122- and OI-71-associated *nle* genes in nonpathogenic *E. coli* strains.

In order to examine the possible spread of the OI-122- and OI-71-associated *nle* genes to other *Enterobacteriaceae*, we have investigated 68 strains of bacteria comprising *Escherichia*, *Cronobacter*, *Yersinia*, *Salmonella*, *Shigella*, *Citrobacter*, *Hafnia*, *Klebsiella*, and *Proteus* species. Except for the two strains of *Shigella dysenteriae* type 1 (stx_1), the *Shigella sonnei* strain CB7888 (stx_1), and the *Citrobacter rodentium* strain CB10835 (*eae*, *nleE*, *nleA*) (data not shown), all other *Enterobacteriacae* isolates tested negative for stx_1, stx_2, *eae*, *ehxA*, and the *nle* genes (Table 4).

TABLE 4. Virulotyping of strains that tested negative for the *eae* and *nle* genes

Serotype	No. tested	*stx* pattern	Species[a]	*ehxA*
O91:H21	6	1 stx_1, 4 stx_2, 1 stx_1 and stx_2	Atypical EHEC	*ehxA*
O91:H21	2	2 stx_2	Atypical EHEC	
O113:H21	7	7 stx_2	Atypical EHEC	*ehxA*
O113:H21	3	3 stx_2	Atypical EHEC	
O100:H−	5	5 stx_2	STEC	
O105:H18	2	1 stx_2, 1 stx_1 and stx_2	STEC	*ehxA*
O109:H−	1	stx_1	STEC	*ehxA*
O110	2	2 stx_1	STEC	
O111:H10	1	stx_2	STEC	
O113:H4	10	2 stx_2, 8 stx_1 and stx_2	STEC	*ehxA*
O113:H4	4	3 stx_2, 1 stx_1 and stx_2	STEC	
O115:H18	1	stx_1 and stx_2	STEC	*ehxA*
O116:H28	1	stx_1	STEC	*ehxA*
O117	2	1 stx_1, 1 stx_1 and stx_2	STEC	
O118:H12	3	3 stx_2	STEC	
O125	1	1 stx_1 and stx_2	STEC	*ehxA*
O126:H8	1	stx_1	STEC	
O128:H2	1	stx_1 and stx_2	STEC	*ehxA*
O136	3	3 stx_1	STEC	
O138	1	stx_2	STEC	
O139:H1	1	stx_2	STEC	
O139:ND	1	stx_2	STEC	
O141:[H4]	1	stx_2	STEC	
O141:H2	1	stx_1 and stx_2	STEC	
O141ac	1	stx_2	STEC	
O146:H28	1	stx_2	STEC	*ehxA*
O146:H28	4	4 stx_2	STEC	
O146:H8	1	stx_1	STEC	
O147	1	stx_1 and stx_2	STEC	
O149:[H19]	1	stx_1	STEC	*ehxA*
O15:H16	1	stx_2	STEC	
O168:H8	1	stx_2	STEC	
O171:H2	1	stx_2	STEC	
O174:H−	1	stx_2	STEC	
O174:H2	5	1 stx_2, 4 stx_1 and stx_2	STEC	*ehxA*
O174:H21	9	8 stx_2, 1 stx_1 and stx_2	STEC	
O174:H8	1	stx_1 and stx_2	STEC	
O174:H8	1	stx_1 and stx_2	STEC	*ehxA*
O178:H19	2	2 stx_1 and stx_2	STEC	*ehxA*
O2:H27	1	stx_2	STEC	*ehxA*
O21:H−	2	2 stx_2	STEC	*ehxA*
O21:H21	4	4 stx_2	STEC	*ehxA*
O22:H16	2	1 stx_2, 1 stx_1 and stx_2	STEC	
O22:H16	2	2 stx_2	STEC	*ehxA*
O22:H8	2	1 stx_2, 1 stx_1 and stx_2	STEC	
O22:H8	2	2 stx_2	STEC	*ehxA*
O22:Hr	1	stx_1 and stx_2	STEC	*ehxA*
O23:H15	1	stx_1 and stx_2	STEC	*ehxA*
O3	2	2 stx_1	STEC	*ehxA*
O30:H12	1	stx_2	STEC	
O39:H48	1	stx_2	STEC	*ehxA*
O40:H21	1	stx_2	STEC	*ehxA*
O41:H7	1	stx_1	STEC	
O46:H38	2	2 stx_1 and stx_2	STEC	*ehxA*
O48	2	2 stx_1 and stx_2	STEC	*ehxA*
O5	1	stx_1 and stx_2	STEC	*ehxA*
O53	2	2 stx_1 and stx_2	STEC	
O6	8	7 stx_2, 1 stx_1 and stx_2	STEC	
O6:H10	1	stx_2	STEC	*ehxA*
O6:H4	1	stx_1	STEC	
O60	1	stx_1	STEC	
O74:H42	1	stx_1 and stx_2	STEC	*ehxA*
O75:H8	1	stx_1 and stx_2	STEC	*ehxA*
O76	1	stx_1	STEC	*ehxA*
O76:H19	1	stx_1	STEC	*ehxA*
O76:H19	1	stx_1	STEC	
O77	2	stx_2	STEC	*ehxA*

Continued on following page

TABLE 4—*Continued*

Serotype	No. tested	*stx* pattern	Species[a]	*ehxA*
O79	1	stx_1 and stx_2	STEC	*ehxA*
O79:H48	1	stx_1 and stx_2	STEC	*ehxA*
O8:H8	2	1 stx_1, 1 stx_2	STEC	
O8:H19	4	4 stx_2	STEC	
O8:H19	1	1 stx_1 and stx_2	STEC	*ehxA*
O88	1	stx_2	STEC	*ehxA*
O91	1	stx_1 and stx_2	STEC	*ehxA*
O91	5	1 stx_1, 1 stx_2, 3 stx_1 and stx_2	STEC	
O91:H9	1	stx_2	STEC	*ehxA*
O91:H10	3	3 stx_2	STEC	
O96:H19	1	stx_1 and stx_2	STEC	*ehxA*
Or:H12	1	stx_1	STEC	
Or	2	stx_2	STEC	
Ox7:H16	1	stx_2	STEC	
Or:H16	1	stx_2	STEC	*ehxA*
Or:H4	1	stx_2	STEC	
O26:H32	1		ETEC	
O1:K1:H−	1		FEC	
O11:H−	1		FEC	
O121:H10	2		FEC	
O125:H30	1		FEC	
O127	1		FEC	
O15:H1	1		FEC	
O16:K1:H−	1		FEC	
O17:H18	1		FEC	
O18:K1:H7	1		FEC	
O2:H1	1		FEC	
O2:H6	1		FEC	
O2:K1:H7	1		FEC	
O2:H−	1		FEC	
O21:H21	1		FEC	
O25:K5	1		FEC	
O4:H5	4		FEC	
O45:K1:H1	1		FEC	
O46:K1:H31	1		FEC	
O6:K+:H−	1		FEC	
O7:K1:H−	1		FEC	
O75:K5:H−	1		FEC	
O78:H−	1		FEC	
O83:K1:H33	1		FEC	
O86	1		FEC	
Or:H−	1		FEC	
O103:H8	1		EC	
O111:H8	1		EC	
O111:H10	1		EC	
O111:H12	1		EC	
O111:H21	1		EC	
O113:H−	1		EC	
O121:[H45]	1		EC	
O132:H18	1		EC	
O142	2		EC	
O145	2		EC	
O145:H2	1		EC	
O153:H12	1		EC	
O157, O157:[H7−]	12		EC	
O157:H10	1		EC	
O157:H12	1		EC	
O157:H15	1		EC	
O157:H16	1		EC	
O157:H19	1		EC	
O157:H25	1		EC	
O157:H42	1		EC	
O157:H43	1		EC	
O2:H1	1		EC	
O26:H21	1		EC	
O55:H19	1		EC	
O6:H4	1		EC	

Continued on following page

TABLE 4—*Continued*

Serotype	No. tested	*stx* pattern	Species[a]	*ehxA*
O62:H30	2		EC	
O_{NT}:H7	1		EC	
O_{NT}	1		EC	
N/A	7		*Salmonella* sp.	
N/A	1		*Yersinia*	
N/A	3		*Klebsiella*	
N/A	4		*Proteus*	
N/A	1		*Citrobacter*	
N/A	3		*Hafnia*	
N/A	2		*Shigella*	
N/A	1		*C. sakazakii*	

[a] EHEC, enterohemorrhagic *E. coli*; STEC, Shiga toxin-producing *E. coli*; ETEC, enterotoxigenic *E. coli*; FEC, *E. coli* isolated from feces of healthy children; EC, *E. coli*.

DISCUSSION

The emergence of O157 and non-O157 EHEC in severe and epidemic human disease is a global health problem (11). The concept of MRA employing the presence of effector genes to diagnose HUS and identify outbreak-associated EHEC strains (9) has opened up new tools to assess the public health risks associated with STEC strains from food, animals, and the environment. In this study, we have evaluated a low-density DNA array that was designed for this purpose.

The detection systems for the major virulence genes of EHEC, i.e., stx_1, stx_2, *eae*, and *ehxA*, were previously evaluated for their specificity (5). Accordingly, *stx* genes were detected only in STEC, EHEC, *S. dysenteriae* type 1, and the *S. sonnei* strain CB7888, which were previously investigated for Stx production and for *stx* genes with block cycler PCR systems (2, 3, 5, 15). Similar findings were made for the *ehxA* gene. Its prevalences were significantly different between STEC (42.66%) and EHEC (90%). *ehxA* was not present in other *E. coli* strains tested in this study. The *eae* gene was detected in typical EHEC strains, as well as in all EPEC strains and in *C. rodentium* (strain 10835). Distribution of the *nle* genes was found to be closely associated with certain serotypes and intimin genotypes in typical EHEC strains, including the new emerging EHEC strains. Remarkably, *nle* genes carried by the module 2 of the O island OI-122 were detected in all typical and new emerging EHEC. In contrast, the *nle* genes issued from the O island OI-71 were not detected in all isolates of EHEC strains. The presence of *nleF* (72.8%) and *nleA* (79%) was less associated with EHEC than the presence of *nleH1-2* (99.5%).

Our approach allows the identification of new emerging EHEC strains that were recently reported as severe human pathogens. One of these is the EHEC O103:H25 type strain, responsible for a food-borne outbreak of HUS in patients from Norway in 2006 (31). Interestingly, this strain had the same *nle* profile as EHEC O157:H7. O5:H_{NM}, as another emerging EHEC type isolated from beef, dairy products, and human patients with HC (24), also shows the *nle* pattern *ent*/*espL2*, *nleB*, *nleE*, *nleF*, *nleH1-2*, *nleA*. A third type of emerging EHEC O118:H16/H_{NM} (23) shows this same *nle* pattern (*ent*/*espL2*, *nleB*, *nleE*, *nleF*, *nleH1-2 nleA*), which is characteristic for EHEC O157:H7 and most of the typical EHEC strains.

On the other hand, not all EHEC strains possess the complete *nle* pattern investigated in this study. Thus, EHEC strains of serotypes O103:H2 and O145:H28 show a second characteristic *nle* pattern with positive signals for only *ent*/*espL2*, *nleB*, *nleE*, and *nleH1-2* genes. These data are in accordance with the results of Creuzburg and Schmidt, who reported the presence of genetic variants of *nleA* in EHEC O103:H2 (*espI*-like) and in EHEC O145:H28 (*nleA3*, *nleA5*, *nleA6-1*, and *nleA11*) which differ significantly from *nleA* (*Z6024*) (10). A total of 15 *nleA* variants have been described with sequence identities at the amino acid level ranging from 71% to 96% (10). Moreover, we found that new emerging EHEC type O15:H2 and O45:H2 strains possess the same *nle* pattern (*ent*/*espL2*, *nleB*, *nleE*, *nleH1-2*) as EHEC O103:H2 and O145:H28 strains.

We report a number of *stx*-negative, *eae*-positive *E. coli* strains belonging to EHEC-associated serotypes which resemble EHEC strains in terms of their *eae* genotypes and their *nle* gene pattern. *nle* typing could thus be useful to identify EHEC variants which have lost their *stx* genes, as is reported to occur upon subculturing (16). It is also of diagnostic value for identification of the causative agent in HUS patients, since it was reported that many of these excrete *stx*-negative variants of the original EHEC strains in the course of the disease (7). The *nle* genes, in different distributions, were also detected in some EPEC strains. Contrary to the results reported by Creuzburg and Schmidt (10), the EPEC strain E2348/69 (O127:H6) tested positive for *nleA* (*Z6024*) in our study. The fact that EPEC strains carry multiple types of *nle* genes indicates that these effectors might play a role in EPEC-induced diarrhea in infants. The *nle* genes were absent in other species of *Enterobacteriaceae* that are frequently isolated from human feces and in fecal *E. coli* from the stool flora of healthy infants. This was taken as evidence that the *nle* virulotyping is specific and suitable for a rapid identification of human-virulent EHEC and possibly EPEC strains.

Data reported in the present study have shown that the simultaneous detection of stx_1 and/or stx_2, *eae*, and *ehxA* genes, together with some non-LEE effector genes located on PAI OI-71 and OI-122, provides a thorough approach for molecular risk assessment of STEC virulence. In summary, the results indicate that EHEC constitutes a heterogeneous group of pathogens sharing a common core of *nle* virulence determinants but may also harbor various distributions of *nle* genes that are strain or serotype specific. It is noteworthy

that the presence in the same strain of a core of virulence determinants (*eae*, *ent*/*espL2*, *nleB*, *nleE*, and *nleH1-2*) is a strong signature of a human-pathogenic EHEC that can cause life-threatening diseases such as HC and HUS. Detection of these genetic markers in all typical EHEC, called the "gang of five" (6), but also in new emerging EHEC types such as O5:H− (24), O15:H2 (8), O118:H16 (23), and O103:H25 (31), shows the importance of monitoring routinely these markers in STEC isolated from the environment, animals, foods, and humans.

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