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Shiga Toxin, Cytolethal Distending Toxin, and Hemolysin Repertoires in Clinical *Escherichia coli* O91 Isolates▽

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Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains of serogroup O91 are the most common human pathogenic *eae*-negative STEC strains. To facilitate diagnosis and subtyping of these pathogens, we genotypically and phenotypically characterized 100 clinical STEC O91 isolates. Motile strains expressed flagellar antigens H8 (1 strain), H10 (2 strains), H14 (52 strains), and H21 (20 strains) or were H nontypeable (Hnt) (10 strains); 15 strains were nonmotile. All nonmotile and Hnt strains possessed the *fliC* gene encoding the flagellin subunit of the H14 antigen (*fliC*$_{H14}$). Most STEC O91 strains possessed enterohemorrhagic *E. coli hlyA* and expressed an enterohemolytic phenotype. Among seven *stx* alleles identified, *stx*$_{2dact}$, encoding mucus- and elastase-activatable Stx2d, was present solely in STEC O91:H21, whereas most strains of the other serotypes possessed *stx*$_1$. Moreover, only STEC O91:H21 possessed the *cdt*-V cluster, encoding cytolethal distending toxin V; the toxin was regularly expressed and was lethal to human microvascular endothelial cells. Infection with STEC O91:H21 was associated with hemolytic-uremic syndrome ($P = 0.0015$), whereas strains of the other serotypes originated mostly in patients with nonbloody diarrhea. We conclude that STEC O91 clinical isolates belong to at least four lineages that differ by H antigens/*fliC* types, *stx* genotypes, and non-*stx* putative virulence factors, with accumulation of virulence determinants in the O91:H21 lineage. Isolation of STEC O91 from patients' stools on enterohemolysin agar and the rapid initial subtyping of these isolates using *fliC* genotyping facilitate the identification of these emerging pathogens in clinical and epidemiological studies and enable prediction of the risk of a severe clinical outcome.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) strains cause diarrhea and a life-threatening hemolytic-uremic syndrome (HUS) worldwide (23, 44). STEC strains isolated from patients usually possess, in addition to one or more *stx* genes, the *eae* gene, encoding adhesin intimin (7, 11, 16, 25, 26, 41, 49). However, a subset of STEC strains associated with human disease lack *eae*, and among these, strains of serogroup O91 are the most common (2, 7, 35, 37, 47, 48). In Germany during the last 5 years, serogroup O91 accounted for 6.4% to 11.0% of all STEC strains reported from human infections and was therefore the fourth-most-common STEC serogroup (after O157, O26, and O103) isolated (47, 48; http://www.rki.de). However, in contrast to *eae*-positive STEC strains of the three leading serogroups, which cause disease mostly in young children (47), STEC O91 is the most common serogroup isolated from adult patients (48).

Despite their association with human diseases worldwide (7, 9, 11, 13, 14, 30, 35, 37, 38, 40, 47, 48), the spectrum of serotypes of STEC O91 isolates from patients and the pathogenic traits of such strains are poorly understood. Moreover, characteristics of STEC O91 strains which could assist with their isolation from human stools and further subtyping in clinical microbiological laboratories have not been systematically investigated or reported. To gain insight into the serotype composition and putative virulence factors of STEC O91 strains causing human disease and to identify characteristics which can facilitate laboratory diagnosis of these organisms, we determined the motility and flagellar phenotypes, *fliC* types, *stx* genotypes, non-*stx* putative virulence loci, and diagnostically useful phenotypes of 100 clinical STEC O91 isolates. Moreover, we investigated possible associations between bacterial characteristics and clinical infection phenotypes.

MATERIALS AND METHODS

Bacterial strains. A total of 100 STEC O91 strains were isolated between 1997 and 2007 from epidemiologically unrelated patients with HUS (4 strains), bloody diarrhea (9 strains), watery diarrhea without visible blood (78 strains), or abdominal cramps without diarrhea (1 strain) or from asymptomatic carriers (8 strains). The strains were isolated from patients in Germany (96 strains), Austria (2 strains), Finland (1 strain), and Canada (1 strain). The 96 German O91 strains represented all human isolates recovered in the authors' laboratories during the study period for which information about the clinical course of the infection was available and represented all O91 serotypes associated with human diseases in Germany during this period. The O91 strains from the other countries were kindly provided by D. Orth (Austrian Reference Laboratory for Enterohemorrhagic *E. coli*, Innsbruck, Austria), A. Siitonen (the National Public Health Institute, Helsinki, Finland), and M. A. Karmali (Laboratory for Food-Borne Zoonoses, Public Health Agency of Canada, Guelph, Ontario, Canada). A subset of the strains ($n = 35$) was described previously (4, 7, 13, 17, 26, 27, 29, 30). The ages of patients from whom the O91 STEC strains originated ranged from 4 months to 89 years (mean, 26.8 years; median, 18 years). The H-antigen reference strains H8 (H 515b; O103:H8), H10 (E 77a; O35:H10), H14 (F 10018-41; O18ab:H14), and H21 (U 11a-44; O8:H21) were from the collection of the Robert Koch Institute, Wernigerode, Germany.

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Case definition. Patients diagnosed as having diarrhea had three or more semisolid or liquid stools without visible blood per day. Bloody diarrhea was defined as diarrhea where visible blood was noted in the stool. HUS was defined as a case of microangiopathic hemolytic anemia (hematocrit less than 30% with peripheral evidence of intravascular hemolysis), thrombocytopenia (platelet count of less than 150,000/mm^3), and renal insufficiency (serum creatinine concentration greater than the upper limit of the normal range for age) (44). Asymptomatic carriers were apparently healthy individuals without diarrhea; they were included in the study to cover the whole spectrum of clinical outcomes associated with STEC O91 strains.

Phenotyping. Strains were confirmed as *E. coli* using API 20 E (bioMérieux, Marcy l'Etoile, France). To assess motility, strains were inoculated into U tubes containing semisolid proteose peptone medium with 0.4% (wt/vol) agar and 0.1% (wt/vol) glucose, incubated at 37°C, and observed for growth daily. An isolate was considered motile if it passed through the U tube within 10 days. If there was no or only limited growth (less than to the tube bottom) from the inoculation site during this time, the isolate was considered nonmotile (NM). Serotyping was performed using antisera against *E. coli* O antigens 1 to 181 and H antigens 1 to 56 and a microtiter method as described earlier (36). The ability to ferment sorbitol was tested on sorbitol MacConkey agar (SMAC) (Becton Dickinson, Heidelberg, Germany). β-D-Glucuronidase activity was assessed using nutrient agar with 4-methylumbelliferyl-β-D-glucuronide (Becton Dickinson). Resistance to tellurite was determined based on the strains' abilities to grow on cefixime-tellurite (CT)–SMAC (Becton Dickinson); to test the influence of cefixime on growth, the strains were inoculated in parallel on Luria-Bertani (LB) agar with 2.5 μg/ml of potassium tellurite (T-LB agar). Production of enterohemorrhagic *E. coli* (EHEC) hemolysin was identified on enterohemolysin agar (Sifin, Berlin, Germany), and production of α-hemolysin was identified on Columbia blood agar (Heipha, Heidelberg, Germany).

Cell culture assays. Stx production was determined by Vero cell cytotoxicity assay (7). The mucus- and elastase-activatable Stx2d phenotype (Stx2dact) (24, 27) was sought using the elastase activation assay performed with 1 U of porcine pancreatic elastase (EC 3.4.21.36; Calbiochem, Darmstadt, Germany) (7). Production of cytolethal distending toxin V (CDT-V) was determined using Chinese hamster ovary (CHO) cells (21). The toxicity of CDT-V toward human endothelium was tested using human brain microvascular endothelial cells (HBMECs) (42); the cells, freshly seeded in 24-well microtiter plates (2.5×10^3 cells per well), were incubated for 5 days (37°C, 5% CO_2) with sterile strain culture supernatants and examined daily by using a light microscope (Axiovert 100; Zeiss, Jena, Germany). In all assays, cytotoxicity titers (defined as the highest supernatant dilutions that caused the typical effect in 50% of the cells) were expressed as geometric means of titers from three independent experiments. Cell viability was tested by trypan blue exclusion (5). HBMECs incubated 5 days in cell culture medium served as a negative control. In addition, the morphological effects of supernatants of CDT-V-producing strains were compared with those of CDT-V-negative strains.

Genotypic characterization. All strains were verified as *E. coli* O91 using PCR targeting wzy_{O91}, a component of the gene cluster encoding biosynthesis of the O91 antigen (33). Putative virulence genes and *ter* genes, encoding tellurite resistance, were detected using published PCR protocols (4, 6, 10, 12, 17, 18, 32, 39, 41). *fliC* genes were subtyped using HhaI restriction fragment length polymorphism (RFLP) analysis of PCR products amplified with primers FSa1 and rFSa1 (41, 49). *stx* genes were subtyped using a combination of RFLP approaches and allele-specific PCR protocols as described previously (7, 16, 17, 50). The designation stx_{2dact} is used throughout to differentiate the alleles encoding activatable Stx2d in the prototype strain B2F1 (45) from an independently described stx_{2d} gene from strain EH250 (34) (hereinafter designated $stx_{2d\text{-}EH250}$), which encodes nonactivatable toxin (7, 20, 50). The five presently known *E. coli cdt* alleles (*cdt*-I, *cdt*-II, *cdt*-III, *cdt*-IV, and *cdt*-V) (4) were detected by PCR (4). Subtyping of *espP* genes (*espP*α, *espP*β, *espP*γ, and *espP*δ) was performed using allele-specific PCRs (10). A novel *espP* allele identified in this study (*espP*ε) was detected using primers 1326F (5′-CTTCAACAATATCTACATGG-3′) and 1878R (5′-CTGAGATAAATCCGAGACT-3′) in 30 cycles of denaturing (94°C, 30 s), annealing (58°C, 1 min), and extension (72°C, 1 min), followed by a final extension (72°C, 5 min).

Sequence analysis. stx_2 and *espP* genes that could not be subtyped using the strategies described above were amplified using primer pairs 5′-GATGGCGGT CCATTATC-3′ and 5′-ACTGAATTGTGACACAGATTA-3′ (20) and esp-A and esp-B (10), respectively. The resulting amplicons were purified (PCR purification kit; Qiagen, Hilden, Germany) and sequenced using an automated ABI Prism 3130xl genetic analyzer and the ABI Prism BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems, Darmstadt, Germany). Sequences were analyzed using the Ridom TraceEditPro software (Ridom GmbH, Würzburg, Germany), and homology was sought in the EMBL-GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

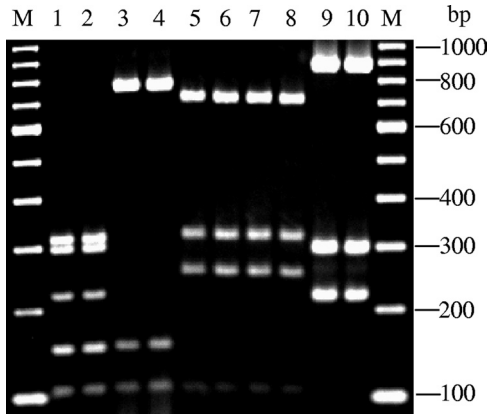

FIG. 1. HhaI *fliC* RFLP patterns of STEC O91 isolates of different serotypes and of Hnt and NM strains compared with *E. coli* H type strains expressing H8, H10, H14, and H21. Lane 1, *E. coli* H8 type strain H 515b (O103:H8), which displays the H8b *fliC* genotype (3); lane 2, STEC O91:H8 (06-03971); lane 3, *E. coli* H10 type strain E 77a (O35:H10); lane 4, STEC O91:H10 (05-06323); lane 5, *E. coli* H14 type strain F 10018-41 (O18ab:H14); lane 6, STEC O91:H14 (00-04243); lane 7, STEC O91:Hnt (01-04459); lane 8, STEC O91:NM (02-03777); lane 9, *E. coli* H21 type strain U 11a-44 (O8:H21); lane 10, STEC O91:H21 (00-04445-2); lane M, 100-bp ladder (Invitrogen).

Statistical analysis. Using Epi-Info version 2002 (Centers for Disease Control and Prevention, Atlanta, GA, and World Health Organization, Geneva, Switzerland), statistical analysis was performed using the χ^2 test and Yates' corrected one-tailed Fisher exact test. P values of <0.05 were considered to be significant.

Nucleotide sequences. The partial sequence of *espP*ε from STEC O91:NM strain 07-00349 was deposited in EMBL-GenBank (accession no. FJ649654).

RESULTS

Serotypes and *fliC* genotypes of STEC O91 isolates. The 100 STEC O91 isolates expressed flagellar antigens H8 (1 isolate), H10 (2 isolates), H14 (52 isolates), H21 (20 isolates), or Hnt (motile, but H-antigen nontypeable) (10 isolates) or were NM (15 isolates). Subtyping of the *fliC* genes, encoding the flagellin subunit, demonstrated four different *fliC* genotypes, which corresponded to the H antigens H8, H10, H14, and H21 of the motile strains (Fig. 1). All 15 O91:NM and all 10 O91:Hnt strains possessed $fliC_{H14}$ (examples in Fig. 1, lanes 7 and 8).

***stx* genotypes.** Seven *stx* alleles and 11 *stx* genotypes were found among the 100 STEC O91 strains (Table 1). $stx_{2d\text{-}EH250}$ occurred only in serotypes O91:H14/Hnt/NM (all $fliC_{H14}$), stx_{2dact} only in serotype O91:H21, and stx_{2c} only in serotype O91:H10 (Table 1). The most frequent *stx* gene among 77 O91:H14/Hnt/NM [$fliC_{H14}$] strains was stx_1, which was found either alone (69 strains [89.6%]) or in combination with $stx_{2d\text{-}EH250}$ (6 strains [7.8%]) (Table 1). In contrast, most (17 of 20 [85.0%]) of the STEC O91:H21 isolates possessed stx_{2dact} either as a single *stx* gene (6 strains [30.0%]) or in combination with stx_1 (1 strain [5.0%]), stx_2 (6 strains [30.0%]), or stx_1 and stx_2 (4 strains [20.0%]) (Table 1). One STEC O91:H21 isolate and the single STEC O91:H8 isolate harbored stx_1 and an unclassified stx_2 variant. The stx_2 variant alleles from the H21 ($stx_{2v\text{-}O91:H21}$) and the H8 ($stx_{2v\text{-}O91:H8}$) strains differed in two nucleotides and displayed 99% identity at the nucleotide and

TABLE 1. *stx* genotypes of STEC O91 isolates belonging to different serotypes

stx genotype[a]	No. of strains of STEC serotype:			
	O91:H8 (n = 1)	O91:H10 (n = 2)	O91:H14/Hnt/NM[b] (n = 77)	O91:H21 (n = 20)[c]
stx_1	0	1	69	0
stx_1 $stx_{2d\text{-}EH250}$	0	0	6	0
$stx_{2d\text{-}EH250}$	0	0	2	0
stx_2	0	0	0	1
stx_2 stx_{2c}	0	1	0	0
stx_{2dact}	0	0	0	6
stx_1 stx_{2dact}	0	0	0	1
stx_2 stx_{2dact}	0	0	0	6
stx_1 stx_2 stx_{2dact}	0	0	0	4
stx_1 $stx_{2v\text{-}O91:H21}$	0	0	0	1
stx_1 $stx_{2v\text{-}O91:H8}$	1	0	0	0

[a] *stx* genotyping was performed as described previously (7, 16, 17, 50), and the stx_2 variants ($stx_{2v\text{-}O91:H21}$ and $stx_{2v\text{-}O91:H8}$) were identified by sequence analysis of nontypeable *stx* genes. stx_{2dact} encodes Stx2d activatable by intestinal mucus and elastase (7, 24, 27); $stx_{2d\text{-}EH250}$ (34) encodes nonactivatable toxin (7, 20, 50).
[b] All Hnt and NM strains possessed $fliC_{H14}$.
[c] One strain lost the stx_2 gene after isolation but before subtyping.

TABLE 2. Comparison of non-*stx* virulence loci in STEC O91 isolates belonging to different serotypes

Gene[a]	No. (%) of strains of STEC serotype:			
	O91:H8 (n = 1)	O91:H10 (n = 2)	O91:H14/Hnt/NM[b] (n = 77)	O91:H21 (n = 20)
cdt[c]	0	0	0[d]	14 (70.0)[d]
subAB[e]	0	0	10 (13.0)	1 (5.0)
EHEC *hlyA*	1 (100.0)	0	55 (71.4)	19 (95.0)
saa	1 (100.0)	0	10 (13.0)[f]	20 (100.0)[f]
iha	1 (100.0)	1 (50.0)	75 (97.4)	20 (100.0)
$lpfA_{O26}$	1 (100.0)	0	76 (98.7)	16 (80.0)
$lpfA_{O113}$	1 (100.0)	1 (50.0)	73 (94.8)	19 (95.0)
espI	0	1 (50.0)	10 (13.0)	0
espP	1 (100.0)[g]	0	49 (63.6)[g,i]	1 (5.0)[h,i]
ter[j]	0	0	0	1 (5.0)

[a] The following genes encode the indicated proteins or activity: *cdt*, cytolethal distending toxin (CDT) (21, 22); *subAB*, subtilase cytotoxin (32); EHEC *hlyA*, EHEC hemolysin (1); *saa*, STEC autoagglutinating adhesin (Saa) (31); *iha*, iron-regulated gene A homologue adhesin (Iha) (43); $lpfA_{O26}$ and $lpfA_{O113}$, major subunits of long polar fimbriae of STEC O26 and O113, respectively (12, 46); *espI*, serine protease EspI (39); *espP*, serine protease EspP (10); and *ter* cluster, tellurite resistance.
[b] All Hnt and NM strains possessed $fliC_{H14}$.
[c] All presently known *E. coli cdt* alleles (*cdt*-I, *cdt*-II, *cdt*-III, *cdt*-IV, and *cdt*-V) were tested (4); all STEC O91:H21 isolates contained the *cdt*-V cluster (21).
[d] STEC O91:H21 versus STEC O91:H14/Hnt/NM [$fliC_{H14}$]: $P < 0.0001$, Yates' corrected $\chi^2 = 45.35$; $CI_{95} = 59.9$ to 1,227.0.
[e] Genes encoding both the A and B subunits of the subtilase cytotoxin were present.
[f] STEC O91:H21 versus STEC O91:H14/Hnt/NM [$fliC_{H14}$]: $P < 0.0001$; $\chi^2 = 30.31$; $CI_{95} = 7.65$ to 18.24.
[g] All strains possessed *espP*ε.
[h] *espP*β.
[i] STEC O91:H21 versus STEC O91:H14/Hnt/NM [$fliC_{H14}$]: $P < 0.0002$; Yates' corrected $\chi^2 = 13.48$; $CI_{95} = 2.6$ to 349.7.
[j] All *ter* genes (*terZABCDEF*) were absent in all *ter*-negative strains, and all were present in the *ter*-positive strain.

amino acid levels to the prototype stx_2 gene and Stx2 protein from STEC O157:H7 strain EDL933 (GenBank accession no. AF125520). Each of the stx_2 variant alleles was 98% identical in nucleotide sequence to each of two stx_{2dact} alleles of the prototype Stx2dact strain B2F1 (45) (GenBank accession no. AF479828 and AF479829) and displayed 98% and 99% identity at the amino acid level to the respective proteins. However, at positions 291 and 297, where serine and glutamic acid, respectively, required for elastase activation, occur in the mature A subunit of Stx2dact (28), phenylalanine and lysine, respectively, were found in each of the STEC O91 Stx2 variants, similar to the result found with Stx2 (28).

Non-*stx* loci of interest. Strains of different serotypes within the O91 serogroup differed by the spectrum of putative non-*stx* virulence genes. The *cdt* cluster, specifically *cdt*-V, encoding CDT-V (4, 21), was found only in STEC O91:H21 strains, being present in the majority (70.0%) of such strains (Table 2); none of the 100 O91 strains contained any of the other four *E. coli cdt* alleles (4). Moreover, all STEC O91:H21 strains possessed *saa*, which encodes the STEC autoagglutinating adhesin (Saa) (31). This locus was also present in the only O91:H8 strain but was significantly less frequently identified in STEC O91:H14/Hnt/NM strains [$fliC_{H14}$] (13.0%) than in STEC O91:H21 strains ($P < 0.0001$) (Table 2). In contrast, the strains with $fliC_{H14}$ contained *espP*, encoding the serine protease EspP (10), significantly more frequently than the O91:H21 strains ($P < 0.0002$) (Table 2). All 49 *espP*-positive O91:H14/Hnt/NM [$fliC_{H14}$] strains harbored a novel *espP* allele, which possessed in its sequenced 858-bp stretch 90% nucleotide sequence identity to the four *espP* alleles identified previously by our group (*espP*α, *espP*β, *espP*γ, and *espP*δ) (10). This allele, which we term *espP*ε (GenBank accession no. FJ649654), was also present in the only O91:H8 strain but was absent from STEC O91:H21 strains (Table 2). Unlike STEC O91:H21 strains, a subset of O91:H14/Hnt/NM [$fliC_{H14}$] strains also contained *espI*, encoding another serine protease, EspI (39). The presence of *espI* correlated 100% with the presence of *saa* and the *subAB* operon, encoding the subtilase cytotoxin (32), but except in one strain, *espI* did not occur together with *espP*. None of the STEC O91 strains contained *eae*, but most, regardless of serotype, possessed genes encoding other putative adhesins, including iron-regulated gene A homologue adhesin (Iha) (43) and long polar fimbriae identified originally in STEC O26 (46) and STEC O113 (12) strains, respectively. The *sfp* cluster, encoding Sfp fimbriae, regularly found in sorbitol-fermenting STEC O157:NM (18) and recently identified also in STEC O165:H25/NM (8) strains, was absent from all STEC O91 strains. Most O91:H21 and O91:H14/Hnt/NM [$fliC_{H14}$] strains, as well as the only O91:H8 strain, possessed EHEC-*hlyA*, encoding EHEC hemolysin (1), which was absent from both O91:H10 strains (Table 2).

Phenotypes. All but one strain (O91:H10) fermented sorbitol, and all produced β-D-glucuronidase. All but two of 75 strains that possessed the EHEC-*hlyA* gene (Table 2) expressed the enterohemolytic phenotype. None of the 100 strains produced α-hemolysin. Except for one O91:H21 strain which possessed the *ter* gene cluster (Table 2) and was tellurite resistant, as demonstrated by its ability to grow on CT-SMAC and T-LB agar, all other strains lacked *ter* genes and failed to grow on both tellurite-containing media. The complete inhibition of the strains' growth on CT-SMAC and T-LB agar demonstrates that tellurite, and not cefixime, is the growth-inhibiting component in CT-SMAC.

Stx production and elastase activation. Sterile supernatants of all but one strain (an O91:H21 strain, which lost stx_2 after

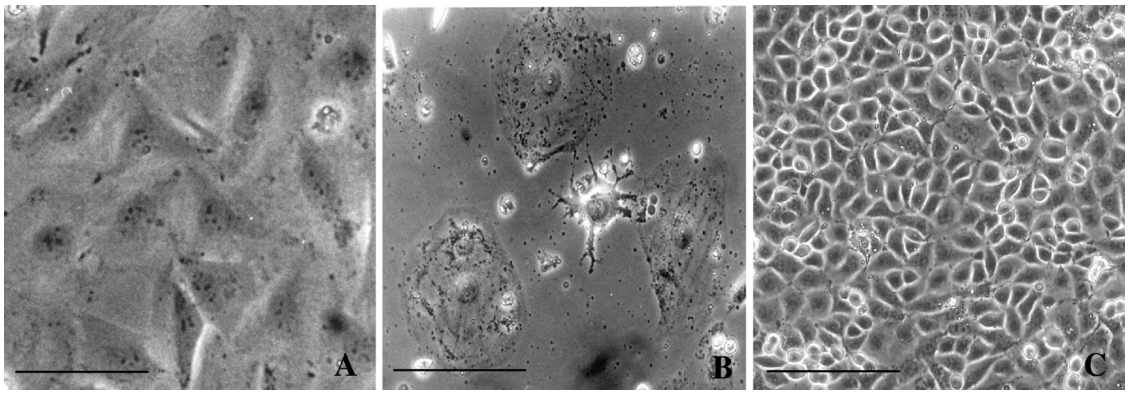

FIG. 2. Photomicrographs of HBMECs after 3 days (A) and 5 days (B) of incubation with supernatant of a CDT-V-producing STEC O91:H21 strain. (C) Control HBMECs incubated 5 days in cell culture medium. Bars, 200 μm.

isolation) (Table 1) were toxic to Vero cells (geometric means of titers, 20 to 4,096; median, 1,024). There were no significant differences in Stx titers between strains of different serotypes. However, the six O91:H21 strains that harbored stx_{2dact} as the only *stx* allele had significantly lower titers (geometric means of titers, 20 to 256; median, 95) than strains of the other *stx* genotypes (geometric means of titers, 128 to 4,096; median, 1,024). Preincubation with elastase resulted in an 8- to 16-fold (median, 12-fold) increase in cytotoxicity titers displayed by the six stx_{2dact}-only strains, demonstrating that all these strains expressed the activatable Stx2d.

CDT-V expression and injury to endothelial cells. Supernatants of all 14 O91:H21 strains that possessed the *cdt*-V cluster caused a characteristic slow-distention effect (21, 22) on CHO cells (geometric means of titers, 8 to 64; median, 16) (data not shown), demonstrating that all produced active CDT-V. Moreover, the CDT-V-containing supernatants caused progressive distention of cultured HBMECs until day 5 after exposure (geometric means of titers, 4 to 32; median, 8) (Fig. 2) which was accompanied by a stepwise loss of cell viability as demonstrated by a trypan blue exclusion test (data not shown). The HBMEC distention caused by CDT-V was clearly distinguishable from Stx-mediated cytotoxicity and was not displayed by supernatants of the six CDT-V-negative, Stx-positive strains.

Disease association. Four of 20 (20.0%) strains of serotype O91:H21 but none of 80 strains of the other (non-H21) serotypes originated from patients with HUS ($P = 0.0015$; $\chi^2 = 11.07$; 95% confidence interval [CI_{95}] = 2.4 to 575.5). Significant association with HUS ($P = 0.002$; $\chi^2 = 10.60$; $CI_{95} = 2.3$ to 554.4) was also observed when only the two most frequent lineages, O91:H21 and O91:H14/Hnt/NM [$fliC_{H14}$], were compared. The O91:H21 strains were also more frequently isolated than the non-H21 strains from patients with bloody diarrhea without HUS (3 of 20 [15.0%] versus 5 of 80 [6.5%]; all $fliC_{H14}$), but this difference was not significant ($P = 0.11$; $\chi^2 = 1.63$; $CI_{95} = 0.6$ to 15.6). Most (75 of 80 [93.8%]) of the non-H21 strains were recovered from patients with nonbloody diarrhea ($n = 67$) or from individuals with asymptomatic infection ($n = 8$ [all $fliC_{H14}$]).

DISCUSSION

Although STEC O91 strains have been isolated in multiple studies worldwide (2, 7, 9, 11, 13, 14, 35, 37, 38, 40, 47, 48), there are presently no systematic analyses of the structure and components of this serogroup. In our characterization of a large collection of STEC O91 clinical isolates originating mostly in Germany, we identified two dominant serotypes, O91:H14 and O91:H21. Two additional serotypes, O91:H8 and O91:H10, are rare causes of human diseases in Germany, and they warrant comment. Whereas STEC O91:H10 strains have been isolated from patients in other countries (2, 9, 14, 35), the STEC strain of serotype O91:H8 isolated in this study from a patient with nonbloody diarrhea has not been, to our knowledge, associated with human disease previously (2). On the other hand, STEC serotype O91:H7, reported as a cause of diarrhea in Argentina (38), was not found in Germany either during this or earlier studies (37, 47), suggesting possible differences in geographic distribution of particular STEC O91 lineages.

Our data provide a rational basis for identifying STEC O91 strains in human stool specimens and for further subtyping of isolates. Specifically, all strains analyzed in this study possessed *stx* genes upon isolation and secreted Stx in sufficient amounts and could therefore be detected in primary stool cultures by commonly applied PCR *stx* screening and/or Stx enzyme immunoassays (23, 25, 37). The isolation of STEC O91 strains from stool samples positive in the screening procedures using SMAC and CT-SMAC (commonly used for the isolation of *E. coli* O157:H7) (44) is hindered by the ability of the vast majority of such strains to ferment sorbitol and by their tellurite susceptibility. However, most (73.0%) STEC O91 isolates produced EHEC hemolysin, a potent cytolysin of red blood cells (1), and expressed an enterohemolytic phenotype on enterohemolysin agar, which distinguishes these strains from *E. coli* found in physiological intestinal flora. Therefore, enterohemolysin agar can be used for isolation of many STEC O91 strains from *stx*/Stx-positive primary stool cultures. STEC O91:H10 strains and the subset of EHEC hemolysin-negative STEC O91 strains of the other serotypes (Table 2) can be isolated using colony hybridization with *stx* probes (25).

O:H serotyping is an important early step in subtyping of clinical *E. coli* isolates. However, with 25 of 100 STEC O91 isolates in this study, this method failed because these strains possessed an H antigen which was nontypeable ($n = 10$) or they were NM ($n = 15$) (Table 1). Importantly, subtyping of *fliC* genes using PCR-RFLP allowed us to classify these strains as members of the H14 lineage; this demonstrates the utility of *fliC* genotyping for a rapid subtyping of O91 clinical isolates, as has been shown for NM *E. coli* O26, O111, O145, and O157 strains (6, 15, 41, 49). Moreover, even in motile strains, *fliC* typing, which can be accomplished within 24 h of isolation, accelerates the exact identification of the strain's serotype, which is important for the assessment of the risk of a severe clinical outcome (HUS) and for epidemiological investigations. Notably, *fliC* subtyping demonstrates that STEC O91 strains associated with human disease are more heterogeneous than strains of any other STEC clade that contains a single *fliC* type (STEC O157 and STEC O26) (6, 15) or two (STEC O145) (41) or three (STEC O111) (49) *fliC* types.

The different *fliC* lineages within the O91 serogroup differ in their *stx* genotypes and particular non-*stx* putative virulence loci and in their virulence. Specifically, strains of serotype O91:H21 which were in our study associated with HUS are the only STEC O91 organisms which possess and express genes encoding Stx2dact. This is in accordance with our previous observation that expression of this Stx phenotype is significantly associated with the virulence of *eae*-negative STEC (7), possibly as a result of Stx2d activation by the intestinal elastase in vivo. Moreover, most STEC O91:H21 strains produced CDT-V, which was absent from strains of all other serotypes. This CDT, which we originally identified in STEC O157:NM and O157:H7 strains (19, 21), is a genotoxin and cyclomodulin which causes DNA damage, cell cycle arrest, and ultimately the death of human microvascular endothelial cells (5). Here we demonstrate that CDT-V produced by STEC O91:H21 strains also causes irreversible injury to the human microvascular endothelium, as demonstrated using a model of HBMECs. Thus, the accumulation in STEC O91:H21 strains of virulence factors that can irreversibly injure microvascular endothelial cells, which are major targets as HUS evolves (44), might contribute to the potential of these strains to cause HUS. This is supported by the finding that STEC O91:H14/Hnt/NM [$fliC_{H14}$] strains, which all lack Stx2dact and CDT-V, were not associated with HUS in our or other studies (37, 40). Notably, in contrast to STEC O91:H21 strains, the majority of STEC O91:H14/Hnt/NM [$fliC_{H14}$] strains harbor the gene encoding a novel EspP, EspPε. Further studies are necessary to determine if and how this molecule might contribute to the virulence of such strains.

In conclusion, STEC O91 strains are a heterogeneous group of organisms which belong to at least four lineages that differ by H antigens/*fliC* types, *stx* genotypes, spectra of non-*stx* putative virulence factors, and levels of pathogenicity to humans, with accumulation of virulence-associated determinants in the O91:H21 lineage. Using enterohemolysin agar for isolation of O91 STEC from stool samples, rapid initial subtyping of the isolates by *fliC* RFLP, and subsequent analysis of virulence profiles make it possible to identify these emerging pathogens in clinical and epidemiological studies and to predict the risk of a severe clinical outcome.

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