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Serotyping, stx_2 Subtyping, and Characterization of the Locus of Enterocyte Effacement Island of Shiga Toxin-Producing *Escherichia coli* and *E. coli* O157:H7 Strains Isolated from the Environment in France

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Twenty-seven Shiga toxin-producing *Escherichia coli* (STEC) strains were isolated from 207 *stx*-positive French environmental samples. Ten of these strains were positive for stx_1, and 24 were positive for stx_2 (10 were positive for $stx_{2vh\text{-}a}$ or $stx_{2vh\text{-}b}$, 19 were positive for stx_{2d}, and 15 were positive for stx_{2e}). One strain belonged to serotype O157:H7, and the others belonged to serogroups O2, O8, O11, O26, O76, O103, O113, O121, O141, O166, and O174. The environment is a reservoir in which new clones of STEC that are pathogenic for humans can emerge.

Shiga toxin-producing *Escherichia coli* (STEC) can survive over long periods in slurries, farmyard manure, and sludge as well as on pasture land and in associated water systems. This observation has important implications for the spread of STEC to crops by direct application of manure or irrigation with infected water or the transmission of these strains directly to humans by contact with animals or contaminated soil (17, 25). Data on the presence of STEC in sewage or polluted water and slurries are limited and difficult to compare (11, 14, 16).

In contrast to a previous study that determined STEC prevalence in manure, slurries, and sewage sludge in France (24), the objective of the present work was to characterize STEC isolates. The detection of the virulence genes and the stx_2 subtypes enabled some speculation about the public health significance of STEC. The study of serotypes combined with that of the locus of enterocyte effacement island (LEE) allowed a better appreciation of the heterogeneity of the isolates and helped to determine whether the environment could be one of the main reservoirs in which new clones pathogenic for humans could emerge.

Four different types of sources were sampled: municipal sewage sites, waste storage lagoons, pig farms, and dairy cattle herds. Nine hundred eighty-eight samples were investigated: 95 farmyard manure samples, 201 bovine feces samples, 144 slurry samples, 10 compost samples, and 538 samples from outflows of sewage wastewater treatment plants. Twelve samples were taken from each source, and each source was sampled only once. The samples were collected over 1 year from two different geographical regions that are representative of France: Bourgogne and Rhone-Alpes. The bacterial enrichment procedure, the detection and isolation of STEC strains, and the identification of the isolates were performed as described by Vernozy-Rozand et al. (24).

All STEC strains were investigated for motility and biochemical properties (such as fermentation of sorbitol in 24 h, β-D-glucuronidase activity, and enterohemolytic phenotype) and were serotyped for their O and H antigens as previously described (3, 15, 18). The Vero cell toxicity assay was performed according to a protocol described previously (4, 5). Vero cells were cultivated in Dulbecco's modified Eagle medium (Gibco BRL). Genetic characterization of the isolates with regard to the presence of stx_1, stx_2, subtypes of stx_2, and types of *eae*, *tir*, *espA*, *espB* and the insertion of the LEE at *selC*, *pheU*, or *pheV*, was performed as previously described (1, 2, 8, 9, 21; V. Livrelli, Y. Bertin, N. Pradel, N. Blanchet, B. Joly, and C. Martin, unpublished data).

Twenty-one percent (207 of 988) of the samples were *stx* positive by PCR. Samples from clarifiers of waste storage lagoons and wastewater treatment plants had contamination rates of 80 and 48%, respectively. These results are in accordance with those obtained by Holler et al. (13), who sampled the inflow and outflow of sewage treatment plants in northern Germany and found 90% of the samples to be positive for stx_1 or stx_2.

We isolated 27 STEC strains from the 207 *stx*-positive samples (13%) by colony hybridization with stx_1 and stx_2 gene probes. Three of the 27 STEC isolates belonged to serogroups O26, O103, and O157. The other serotypes are given in Table 1. Serogroups O26, O55, O111, O103, and O157:H7 are the

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TABLE 1. Phenotypic and genotypic characterization of isolates[a]

Origin of strain	Strain no.	Sample type	Serotype[b]	Hemolysin phenotype[c]	Status regarding:					stx_2 variant(s)
					eae gene	*ehxA* gene	Verocytotoxin production	stx_1 gene	stx_2 gene	
Wastewater treatment plant	V1	Slurry	O166:H28	ehly	N	P	P	N	P	stx_{2e}, $stx_{2d\text{-}Ount}$
	V2	Clarifier	O113:H4	N	P	P	P	N	P	$stx_{2\text{-}NV206}$, stx_{2d}
	V3	Slurry pit	O2:H27	N	N	P	P	N	P	$stx_{2\text{-}EDL933}$, stx_{2d}
	V4	Aerator	O76:H19	ehly	P	P	P	P	P	stx_{2e}, $stx_{2d\text{-}Ount}$
	V5	Clarifier	O26:H11	ehly	P	P	P	P	N	N
	V6	Sludge	O76:H19	ehly	N	P	P	P	P	stx_{2e}, $stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V7	Clarifier	O76:H19	ehly	P	P	P	P	P	stx_{2e}, $stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V8		O76:H19	ehly	N	P	P	N	P	$stx_{2\text{-}EDL933}$
	V9	Aerator	O157:H7	ehly	P	P	P	P	P	$stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V10	Clarifier	O11:H43	N	N	N	P	N	P	N
Pig farms	V11	Feces	Ont:H4	alpha	N	N	P	N	P	stx_{2e}
	V12	Feces	O141:H4	alpha	N	N	P	N	P	stx_{2e}
	V13	Feces	O8:H19	−N	N	N	P	N	P	$stx_{2\text{-}NV206}$, stx_{2e}, stx_{2d}
	V14	Feces	O103:H2	ehly	P	P	P	N	P	stx_{2e}, stx_{2d}
	V15	Feces	Ont:H19	N	N	P	P	P	P	$stx_{2vh\text{-}b}$, stx_{2e}, $stx_{2d\text{-}Ount}$
	V16	Feces	O121:H−	N	N	N	P	N	P	stx_{2e}
Dairy cattle herd	V17	Feces	Ont:H38	N	P	P	P	N	P	$stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V18	Feces	Orough:H25	ehly	P	P	P	N	P	stx_{2e}, $stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V19	Feces	Orough:H25	alpha	P	P	P	N	P	$stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V20	Feces	O113:H4	N	N	N	P	N	P	$stx_{2\text{-}NV206}$, stx_{2e}, stx_{2d}
	V21	Manure	O174:H21	N	N	N	P	N	P	$stx_{2vh\text{-}b}$, stx_{2e}, $stx_{2d\text{-}Ount}$
	V22	Manure	O174:H21	N	N	N	P	N	P	$stx_{2vh\text{-}b}$, stx_{2e}, $stx_{2d\text{-}Ount}$
	V23	Manure	O174:H21	ehly	N	P	P	P	P	stx_{2e}, $stx_{2\text{-}EDL933}$, stx_{2d}
	V24	Slurry pit	O8:H19	ehly	N	P	P	P	N	N
	V25		Ont:H8	N	N	P	P	P	N	N
	V26	Feces	O174:H−	N	N	N	P	N	P	$stx_{2vh\text{-}a}$, $stx_{2d\text{-}Ount}$
	V27	Manure	O8:H19	ehly	N	P	P	P	P	$stx_{2\text{-}EDL933}$, stx_{2d}

[a] P, positive; N, negative.
[b] Orough, rough; Ont, O type not corresponding to any serogroup between O1 and O174.
[c] ehly, hemolysis after a 22-h incubation step; alpha, hemolysis after 3- to 4-h incubation step.

STEC serogroups isolated most frequently from French human patients with hemorrhagic colitis and hemolytic uremic syndrome (12). Twenty-four STEC strains possessed the stx_2 gene, and 10 possessed the stx_1 gene. Seven strains (26%) possessed both stx_1 and stx_2. Seven (26%) strains were *eaeA* positive, and 18 (66%) were *ehxA* positive. Nine STEC strains carried three different virulence factors (*stx*, *eae*, and *ehxA*) (Table 1). These strains should be considered potentially pathogenic for humans.

Epidemiological studies, together with in vivo and in vitro experiments, have revealed that stx_2 is the most important virulence factor associated with severe human disease (6, 19). The genes encoding these toxins (*stx* genes) are encoded by lambdoid bacteriophages. At least 12 stx_2 gene variants have been described (1, 2, 23). Some of these variants, which are associated with STEC strains isolated from specific hosts such as sheep (stx_{2d}) and pigs (stx_{2e}), are probably less pathogenic for humans (10, 26). The type of variant could thus reflect not only the origins and relationships but also the virulences of the different STEC strains.

In our collection of environmental STEC strains isolated in France, the most frequent stx_2 subtype was stx_{2d} (70% of the isolates), followed by stx_{2e} (55%) and $stx_{2vh\text{-}a}$ or $stx_{2vh\text{-}b}$ (37%). The stx_{2e} variants were isolated from pig samples but also from a dairy cattle herd, a finding which is not in accordance with data given in the literature (7, 20) and suggests the spread of STEC from pigs to cattle.

The presence of more than one stx_2 gene, which was observed in 70% of our STEC isolates, could contribute to the level of toxin production and thus to the virulence of environmental isolates.

In a previous study, an absolute correlation between the presence of *eae* and the presence of the other genes of the LEE was found (2). The LEE gene was not detected in *eae*-negative strains. On the basis of these findings, we used the *eae* gene to screen for LEE-positive STEC isolates. EspA, EspB, Tir, and intimin proteins encoded by different LEEs show high degrees of sequence polymorphism. To date, 10 distinct variants of *eae* and three variants of *espA*, *espB*, and *tir* have been described (22, 27). Previous studies have indicated that some serotypes are highly associated with a particular intimin variant (8, 9). These associations were confirmed in our study; the O157 serogroup is associated with *eae*γ, the O26 serogroup is associated with *eae*β, and the O103 serogroup is associated with *eae*ε. No other *eae* variants, including *eae*α, were found. In this study, we detected only three distinct pathotypes, and except for the O103 STEC strain, *tir*, *espA*, and *espB* genes were always of the same type within a pathotype. We also observed a high degree of heterogeneity among the seven *eae*-positive isolates from our collection relative to the LEE integration site

TABLE 2. Characterization of the LEE

Origin of strain	Strain no.	Serotype	Subtype of gene				PCR analysis result for[a]:								
							selC site			*pheU* site			*pheV* site		
			eae	*espA*	*espB*	*tir*	Intact (K260/K295)	Right side (K260/K255)	Left side (K295/K296)	Intact (K913/K916)	Right side		Left side (P54/P15)	*pheV* (913/914)	Intact (*pheV*/*pheVR*)	Right side (P64/P86)
											K913/K917	P119/P32				
Wastewater treatment plant	V4	O76:H19	β	β	β	β	N	N	N	N	P	N	N	N	N	P
	V5	O26:H11	β	β	β	β	N	N	N	N	P	N	N	N	N	P
	V9	O157:H7	γ	γ	γ	γ	N	P	P	P	N	N	N	P	N	P
Pig farm	V14	O103:H2	ε	βv	β	β	P	N	N	N	N	N	N	N	N	N
Dairy cattle herd	V17	Ont:H38	β	β	β	β	N	N	N	N	P	N	N	N	N	N
	V18	Orough:H25	β	β	β	β	N	N	N	N	P	N	P	N	N	P
	V19	Orough:H25	β	β	β	β	N	N	N	N	P	N	N	N	N	N

[a] N, negative; P, positive. K260, K295, K255, K296, K913, K916, K917, P119, P32, P54, P15, 913, 914, *pheVF*, *pheVR*, P64, and P86 are primers.

as well as the sequences of the right and left ends of the LEE (Table 2).

This study demonstrated that the environment is a reservoir in which new clones of STEC that are pathogenic for humans could emerge. A comparison with STEC strains isolated from patients with hemolytic uremic syndrome will be undertaken in the near future.

Our results emphasize that appropriate handling and use of manure, slurry, and sewage sludge are necessary so that contamination of the environment and food by STEC can be prevented.

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