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Characteristics of Emerging Human-Pathogenic *Escherichia coli* O26:H11 Strains Isolated in France between 2010 and 2013 and Carrying the stx_{2d} Gene Only

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Strains of *Escherichia coli* O26:H11 that were positive for stx_2 alone ($n = 23$), which were not epidemiologically related or part of an outbreak, were isolated from pediatric patients in France between 2010 and 2013. We were interested in comparing these strains with the new highly virulent stx_{2a}-positive *E. coli* O26 clone sequence type 29 (ST29) that has emerged recently in Europe, and we tested them by multilocus sequence typing (MLST), stx_2 subtyping, clustered regularly interspaced short palindromic repeat (CRISPR) sequencing, and plasmid (*ehxA*, *katP*, *espP*, and *etpD*) and chromosomal (Z2098, *espK*, and *espV*) virulence gene profiling. We showed that 16 of the 23 strains appeared to correspond to this new clone, but the characteristics of 12 strains differed significantly from the previously described characteristics, with negative results for both plasmid and chromosomal genetic markers. These 12 strains exhibited a ST29 genotype and related CRISPR arrays (CRISPR2a alleles 67 or 71), suggesting that they evolved in a common environment. This finding was corroborated by the presence of stx_{2d} in 7 of the 12 ST29 strains. This is the first time that *E. coli* O26:H11 carrying stx_{2d} has been isolated from humans. This is additional evidence of the continuing evolution of virulent Shiga toxin-producing *E. coli* (STEC) O26 strains. A new O26:H11 CRISPR PCR assay, SP_O26_E, has been developed for detection of these 12 particular ST29 strains of *E. coli* O26:H11. This test is useful to better characterize the stx_2-positive O26:H11 clinical isolates, which are associated with severe clinical outcomes such as bloody diarrhea and hemolytic uremic syndrome.

Enterohemorrhagic *Escherichia coli* (EHEC) is responsible for gastrointestinal diseases such as diarrhea or bloody diarrhea and can lead to hemolytic uremic syndrome (HUS). The most common EHEC serotype associated with human disease is O157:H7. However, a growing number of human EHEC infections are caused by non-O157 EHEC strains (1–4). Among non-O157 EHEC strains, O26:H11 has emerged as the most common serotype associated with severe diarrhea and HUS worldwide (1–6).

EHEC O26:H11 strains are very dynamic; they can undergo frequent genetic rearrangements in their chromosome, virulence plasmids, and pathogenicity islands. They also have the ability to rapidly lose and acquire *stx*-carrying phages (7), which makes them highly adaptable and may account for their global spread. Until recently, EHEC O26:H11 strains isolated from humans mostly harbored Shiga toxin 1 (Stx1) (Stx1a subtype) only or, more rarely, Stx1a associated with the Stx2a subtype. In the middle 1990s, however, a new EHEC O26:H11 clone carrying the Shiga toxin Stx2a subtype alone emerged in Europe (8–18). This new clone has also been observed in South America (19) and in the United States (1). Shiga toxin-producing *E. coli* (STEC) strains carrying the stx_2 gene are usually associated with more severe outcomes (20). Indeed, this new O26:H11 clone appears highly virulent and is significantly associated with HUS (8, 9).

Multilocus sequence typing (MLST) analysis shows that the stx_{2a}-harboring *E. coli* O26:H11 strains are mostly divided into 2 related phylogenetic groups, i.e., sequence type 21 (ST21) (which also contains the EHEC O26:H11 strains harboring stx_{1a} alone or in combination with stx_{2a}) and ST29 (which contains the new highly pathogenic clone carrying stx_{2a} only) (9). The large EHEC plasmids encoding enterohemolysin (*ehxA*), catalase peroxidase (*katP*), serine protease (*espP*), and type II effector (*etpD*) can be found in most EHEC O26:H11 strains (8, 9), and the presence of these specific plasmid virulence determinants can be used to distinguish the 2 clones. ST21 is characterized by the plasmid gene combination *ehxA*+/*katP*+/*espP*+/*etpD*−, while ST29 exhibits the distinctive combination *ehxA*+/*katP*−/*espP*−/*etpD*+ (9).

Chromosomally encoded virulence factors such as the locus of enterocyte effacement (LEE) effectors and some type III secretion system effectors were also found to be conserved in the phylogenetic group ST21 (21). In the context of a molecular risk assessment strategy, we previously described a combination of molecular markers for specific identification of EHEC and EHEC-like O26:H11 strains. Assays for these markers included wzx_{O26}, $fliC_{H11}$, *eae*-β, *stx*, *espK*, and *arcA* single-nucleotide polymorphism (SNP) genotyping (21), as well as a set of PCR tests (SP_O26_C and SP_O26_D) targeting the clus-

Received 8 August 2014 **Returned for modification** 16 September 2014
Accepted 17 November 2014

Accepted manuscript posted online 26 November 2014

Citation Delannoy S, Mariani-Kurkdjian P, Bonacorsi S, Liguori S, Fach P. 2015. Characteristics of emerging human-pathogenic *Escherichia coli* O26:H11 strains isolated in France between 2010 and 2013 and carrying the stx_{2d} gene only. J Clin Microbiol 53:486–492. doi:10.1128/JCM.02290-14.

Editor: N. A. Ledeboer

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doi:10.1128/JCM.02290-14

tered regularly interspaced short palindromic repeat (CRISPR) locus of EHEC O26:H11 (22).

Although they have been observed all over Europe, the stx_2-harboring O26:H11 strains circulating in France have not been thoroughly characterized. In the present study, we analyzed 23 stx_2-harboring *E. coli* O26 strains isolated from different sporadic infections that occurred in France between 2010 and 2013. We characterized their chromosomal and plasmid virulence gene contents to compare them with STEC O26 strains circulating in other countries in Europe. We also performed multilocus sequence typing (MLST), to examine phylogenetic relatedness, and CRISPR typing, to look for genetic diversity among the strains.

MATERIALS AND METHODS

Bacterial strains. The surveillance of STEC infections in France is based on the surveillance of HUS in children <15 years of age. A nationwide surveillance system, relying on voluntary reporting of pediatric HUS cases by the pediatric nephrology units from a network of 31 public hospitals, was set up in 1996. Clinical strains used in this study are from the strain collection of the pediatric Hôpital Robert-Debré (Paris, France), which is the associated national reference laboratory for *E. coli*. Between 2010 and 2013, 46 STEC O26 strains were isolated from HUS in children <15 years of age. Except for 3 household transmissions, all O26-based infections were sporadic, and no food source was identified. During this time period, no O26 outbreak was reported. The 23 STEC O26:H11 strains studied were originally isolated from stool specimens or rectal swabs from patients with HUS in various part of France. They were not epidemiologically related or part of an outbreak. They were selected to include only strains positive for stx_2 alone.

Genetic characterization by real-time PCR. The 23 STEC O26:H11 strains were tested by real-time PCR for the presence of wzx_{O26}, $fliC_{H11}$, stx_1, stx_2, stx_{2a}, *eae*, *eae*-β, *ehxA*, *katP*, *espP*, *etpD*, *arcA* allele 2, *espK*, *espV*, Z2098, O26:H11 CRISPR (assays SP_O26_C, SP_O26_D, and SP_O26_E), and *wecA* (as a genetic marker of *E. coli*), using a Biomark (Fluidigm, San Francisco, CA) or LightCycler Nano (Roche Diagnostics, Meylan, France) thermocycler. All primers and probes were described previously (21–24) except for the probe for *arcA* allele 2, which was modified from the report by Bugarel et al. (21) with locked nucleic acid (LNA)-substituted nucleotides to specifically bind *arcA* allele 2 (arcA2-Taq, 5′-CAAAATTCAGTCCC+G+TGC-3′ [+ indicates LNA-substituted nucleotides]), and SP_O26_E, for which the reverse primer (SP_O26-R, 5′-ATCAACATGCAGCGCGAACG-3′) was used with the previously described SP_O26_D forward primer and probe (22). The primers and probe for stx_{2a} were as follows: stx2a-F, 5′-TTCTGTTAATG CAATGGCGGCG-3′; stx2a-R, 5′-CCAGTATTCTTTCCCGTCAACCTT C-3′; stx2a-Taq, 5′-AATGTGTCATCCTCATTATACTTGG-3′.

CRISPR typing. Sequence polymorphisms of the CRISPR loci in the strains were examined using the nomenclature of CRISPR1 and CRISPR2a (25). The CRISPR loci were amplified by PCR as described previously (22). Amplicons were double-strand sequenced (Eurofins MWG Operon, Courtaboeuf, France) and the CRISPR sequences of the strains were assembled using BioEdit version 7.1.3.0. Analysis of the CRISPR loci was as described by Feng et al. (26).

Multilocus sequence typing. Multilocus sequence typing (MLST) was performed for the 23 strains using seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*), as described by Wirth et al. (27) and modified by Bielaszewska et al. (7). The alleles and sequence types (STs) were assigned in accordance with the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Partial sequencing of stx_2 genes. Partial sequencing of the stx_2 genes of the 7 strains negative for stx_{2a} was performed according to the method described by Scheutz et al. (28), with primers F4 and R1. Nucleotide sequences were assembled using BioEdit version 7.1.3.0 and were analyzed by comparison with the established reference sequences. The nucleotide sequences were translated to amino acids and compared using CLC Sequence Viewer version 7.0.2.

RESULTS

Strains and patients. The 23 STEC O26:H11 strains studied were selected to include only strains positive for stx_2 alone. These strains were isolated mainly from female patients (65%), rather than male patients (35%). The patients' median age at the time of isolation was 16 months (Table 1).

Multilocus sequence typing. In order to better characterize the phylogenetic relationship of these strains and to compare them with the highly virulent new stx_{2a}-harboring clone ST29, we performed MLST analysis (Fig. 1). Two STs were obtained. Seven isolates were grouped in ST21 and 16 isolates in ST29, both from clonal complex 29 (CC29).

Plasmid gene profiles. We characterized the plasmid gene contents of the 23 strains to compare them with STEC O26 strains circulating in other countries in Europe and described by others (8–10). The *ehxA*, *katP*, *espP*, and *etpD* genes were distributed in three different patterns (Table 2). One dominant profile was found in 12 strains (52% of isolated strains), in which all plasmid genes were absent. All strains in this profile belonged to ST29. One combination (lacking only *etpD*) was present in 7 strains, all of which belonged to ST21. The plasmid gene combination previously identified as characteristic of the new clone (*ehxA*+/*katP*-/*espP*-/*etpD*+) was found in 4 strains from ST29.

Virulence gene profiles. We also tested all strains for the presence of a set of genetic markers previously postulated by Bugarel et al. (21) to be specific for EHEC and EHEC-like O26 strains (Table 2). All isolates were confirmed to be *Escherichia coli* through testing for the housekeeping gene *wecA*. All strains also tested positive for wzx_{O26}, $fliC_{H11}$, stx_2, *eae*, and *eae*-β, while they all tested negative for stx_1. Eleven strains (belonging to ST21 and ST29) tested positive for *arcA* allele 2, *espK*, *espV*, Z2098, and SP_O26_C and/or SP_O26_D.

Although positive for wzx_{O26}, $fliC_{H11}$, stx_2, and *eae*-β, 12 strains exhibited peculiar genetic characteristics, as they appeared negative for *arcA* allele 2, *espK*, *espV*, Z2098, and SP_O26_C. Five of these strains were nonetheless positive for SP_O26_D. These 12 strains corresponded to those that were negative for all plasmid genes, and they belonged to ST29.

CRISPR typing. All isolates were subtyped according to the sequences of the CRISPR1 and CRISPR2a loci. The spacer arrangements of CRISPR1 and CRISPR2a loci are shown in Fig. 1.

Five alleles were found for the CRISPR1 locus (21% allele diversity). These alleles are constituted by different modular arrangements of a set of 9 spacers. One CRISPR1 allele (allele 29) was found in 12/23 strains (52% of the strains), all of which belonged to ST29. This CRISPR1 allele was characterized by a reduced number of spacers (5 spacers). The strains carrying this CRISPR1 allele were also the ones in which all plasmid and virulence gene markers were absent. The next most frequent CRISPR1 allele (allele 11, found in reference strain 11368) was found in 6 strains (26%), which belonged to two different STs and plasmid profiles. Among the other less frequent CRISPR1 alleles, two alleles were found twice and one allele was found once.

We found four alleles for the CRISPR2a locus (17% allele diversity). In contrast to CRISPR1, the alleles of CRISPR2a are not variations of the same set of spacers but rather constitute 2

TABLE 1 Characteristics of *E. coli* O26:H11 clinical isolates and patients

Strain	Serotype	stx_1	stx_2	*eae*	*eae*-β	Yr of isolation	Origin	Specimen	Patient sex[a]	Patient age
30993	O26:H11	−	2a	+	+	2010	HUS	Stool	F	2 yr (25 mo)
31131	O26:H11	−	2a	+	+	2010	HUS	Stool	F	22 mo
31132	O26:H11	−	2a	+	+	2010	HUS	Stool	M	14 mo
31493	O26:H11	−	2a	+	+	2010	HUS	Stool	F	11 yr
32802	O26:H11	−	2d	+	+	2011	HUS	Stool	M	9 mo
32876	O26:H11	−	2a	+	+	2011	HUS	Stool	F	2 yr (26 mo)
33116	O26:H11	−	2a	+	+	2011	HUS	Rectal swab	M	13 mo
33344	O26:H11	−	2a	+	+	2011	HUS	Stool	F	3 mo
33618	O26:H11	−	2d	+	+	2012	HUS	Stool	F	16 mo
34130	O26:H11	−	2a	+	+	2012	HUS	Rectal swab	F	13 mo
34195	O26:H11	−	2a	+	+	2012	HUS	Rectal swab	M	2 yr (32 mo)
34586	O26:H11	−	2d	+	+	2012	HUS	Rectal swab	M	8 mo
34620	O26:H11	−	2a	+	+	2012	HUS	Stool	M	2 yr (25 mo)
34629	O26:H11	−	2d	+	+	2012	HUS	Stool	M	2 yr (28 mo)
34760	O26:H11	−	2a	+	+	2012	HUS	Rectal swab	F	12 mo
34827	O26:H11	−	2a	+	+	2012	HUS	Stool	F	14 mo
34870	O26:H11	−	2a	+	+	2012	HUS	Stool	M	13 mo
36079	O26:H11	−	2a	+	+	2013	HUS	Stool	F	2 yr
36084	O26:H11	−	2a	+	+	2013	HUS	Stool	F	2 yr
36293	O26:H11	−	2d	+	+	2013	HUS	Stool	F	7 mo
36348	O26:H11	−	2d	+	+	2013	HUS	Stool	F	19 mo
36493	O26:H11	−	2d	+	+	2013	HUS	Stool	F	4 mo
36708	O26:H11	−	2a	+	+	2013	HUS	Stool	F	4 yr

[a] F, female; M, male.

groups with a common core set of spacers and some unique spacers. The first group contained CRISPR2 alleles 67 and 71, which were found in the 12 strains that contained CRISPR1 allele 29 and in which all plasmid and virulence gene markers were absent. This group was characterized by the presence of a 1,260-bp transposon within the first spacer. These 2 CRISPR2 alleles otherwise differed by the presence of 2 additional spacers in allele 71. The second group, found in 11 strains, contained CRISPR2 allele 4 (found in reference strain 11368) and allele 119, which differed by the additional presence of 3 spacers in allele 4. Together, the CRISPR1 and CRISPR2a allele combinations form 7 CRISPR types (CTs).

Stx genotypes (subtypes). The stx_{2a} subtype was tested in all strains by real-time PCR testing, and 16 strains were confirmed to be positive for stx_{2a}. Surprisingly, 7 strains were found to be negative for stx_{2a} although they were positive for stx_2. To identify the stx_2 subtype(s) of these strains, we determined the partial sequences of the $stxAB_2$ operons of these 7 strains. The sequences were analyzed and compared with the published stx_2 reference sequences (28). Through analysis of the nucleotide sequences, the stx_2 gene for the 7 samples was identified as stx_{2d}. The nucleotide sequences were 100% identical among the 7 samples and were most similar (one nucleotide difference) to that of strain 5905 (O55:H7; GenBank accession no. EF441605)

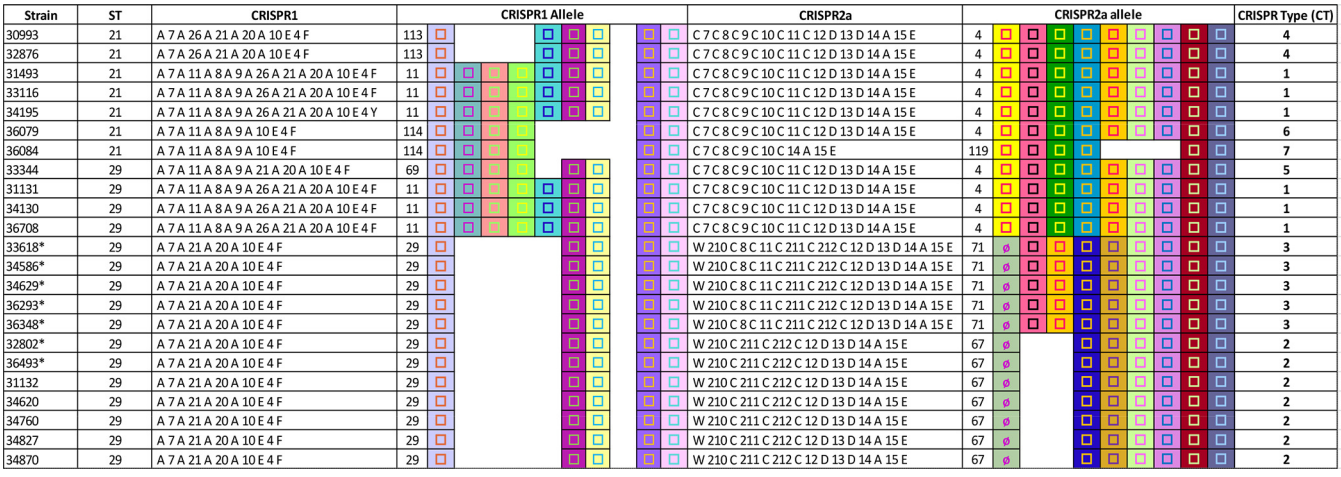

FIG 1 MLST and CRISPR typing of the O26:H11 clinical isolates. In the graphic representation of spacer arrangements in CRISPR1 and CRISPR2a, each unique spacer is represented by a unique combination of the center shape and background color. The shape in the center indicates the spacer length (□, 32 bp; Ø, 1,293 bp). Gaps were introduced to improve the alignment of similar CRISPR arrays. CRISPR1 and CRISPR2a allele numbers are shown in the respective columns to the left of the CRISPR arrays. Each unique combination of CRISPR1 and CRISPR2a alleles was assigned a CRISPR type (CT) number. *, stx_{2d}-positive strains.

TABLE 2 Plasmid and virulence gene profiles of *E. coli* O26:H11 clinical isolates

Strain	stx_{2a}	*ehxA*	*katP*	*espP*	*etpD*	*arcA* allele 2	Z2098	*espK*	*espV*	SP_O26_C	SP_O26_D	SP_O26_E
30993	+	+	+	+	−	+	+	+	+	+	+	−
32876	+	+	+	+	−	+	+	+	+	+	+	−
31493	+	+	+	+	−	+	+	+	+	+	+	−
33116	+	+	+	+	−	+	+	+	+	+	+	−
34195	+	+	+	+	−	+	+	+	+	+	+	−
36079	+	+	+	+	−	+	+	+	+	+	+	−
36084	+	+	+	+	−	+	+	+	+	−	+	−
33344	+	+	−	−	+	+	+	+	+	+	+	−
31131	+	+	−	−	+	+	+	+	+	+	+	−
34130	+	+	−	−	+	+	+	+	+	+	+	−
36708	+	+	−	−	+	+	+	+	+	+	+	−
33618[a]	−	−	−	−	−	−	−	−	−	−	+	+
34586[a]	−	−	−	−	−	−	−	−	−	−	+	+
34629[a]	−	−	−	−	−	−	−	−	−	−	+	+
36293[a]	−	−	−	−	−	−	−	−	−	−	+	+
36348[a]	−	−	−	−	−	−	−	−	−	−	+	+
32802[a]	−	−	−	−	−	−	−	−	−	−	−	+
36493[a]	−	−	−	−	−	−	−	−	−	−	−	+
31132	+	−	−	−	−	−	−	−	−	−	−	+
34620	+	−	−	−	−	−	−	−	−	−	−	+
34760	+	−	−	−	−	−	−	−	−	−	−	+
34827	+	−	−	−	−	−	−	−	−	−	−	+
34870	+	−	−	−	−	−	−	−	−	−	−	+

[a] stx_{2d}-positive strain.

(29). The sequences were translated to amino acids (Fig. 2), confirming the activatable type of the Stx2d toxin with the combined presence of the "activatable tail" in the Stx2A subunit (KSQSLYTTGE) and the END motif at positions 14 to 16 in the Stx2B subunit. As STEC can carry multiple stx_2-subtype genes, we tested the 23 strains for all known stx_2 subtypes using PCR assays described by Scheutz et al. (28). No strain was carrying multiple stx_2-subtype genes. Strains were positive either for stx_{2a} only or for stx_{2d} only.

Development of CRISPR PCR test for detection of CRISPR2a alleles 67 and 71. In order to detect the STEC O26:H11 strains that were negative for the previously published genetic markers *arcA* allele 2, *espK*, *espV*, Z2098, and O26 CRISPR (SP_O26_C), we developed a new real-time PCR assay targeting the CRISPR2a locus of the strains

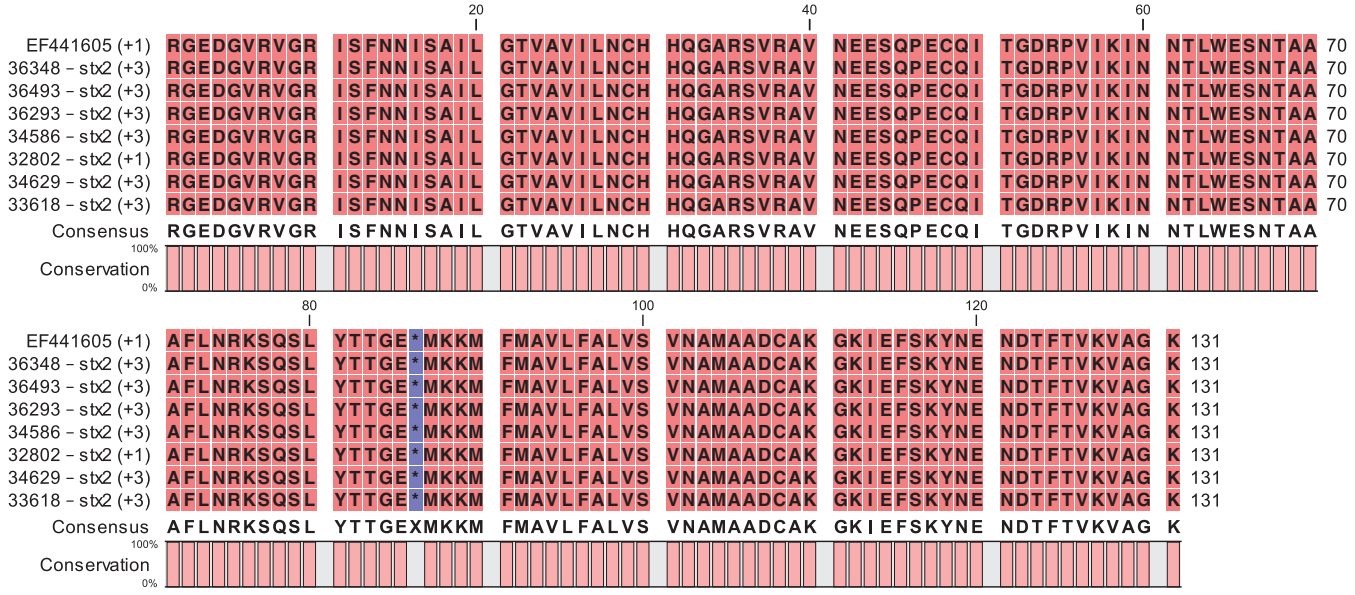

FIG 2 Alignment with reference strain 5905 of the amino acid sequences of the C-terminal ends of the A subunits and the N-terminal ends of the B subunits of the 7 stx_{2a}-negative and stx_{2d}-positive strains. Numbers in parentheses indicate the reading frames used for amino acid translation. Asterisks indicate the separation of the amino acid sequences of subunits A and B of Stx2.

exhibiting CRISPR2a alleles 67 and 71, SP_O26_E. This assay was tested on all strains and, as expected, only strains with CRISPR2a alleles 67 and 71 were found to be positive (Table 2).

DISCUSSION

In France, 122 (in 2010) to 162 (in 2013) pediatric HUS cases were reported each year, 63% to 75% of which were confirmed by bacteriology or serology, between 2010 and 2013. Among these pediatric HUS patients, the majority (51%) were female (mean age, 2.6 years). During this time period, O26 was consistently the second most frequently isolated serogroup in France, representing 15% (in 2010) to 18% (in 2013) of isolated EHEC strains in HUS patients <15 years of age. Prior to the 1990s, the clinical EHEC O26:H11 strains isolated in Europe mainly possessed the stx_1 gene alone or, more rarely, in association with stx_{2a}. Over the past decade, a shift in the prevalence of EHEC O26:H11 strains recovered from humans has been observed. Since the middle 1990s, most of the clinical O26:H11 strains possess the stx_{2a} gene alone or in association with *stx1*, and a new highly pathogenic clone carrying stx_{2a} only has started to emerge in Europe (8–10).

In this study, we characterized the genetic properties of stx_2-harboring O26:H11 clinical strains circulating in France between 2010 and 2013. We confirmed that the new highly pathogenic EHEC O26:H11 clone ST29 is circulating in France and constituted most of the stx_2-harboring EHEC O26:H11 strains studied here, as 16 of the 23 strains appeared to correspond to this clone (ST29). This finding confirms observations made by others in different countries (3, 8–18). However, the characteristics of many of the strains analyzed here differed somewhat from the characteristics described previously.

Indeed, 12 strains were negative for all plasmid genes. This dominant profile (52% of the strains) was reported previously with a much lower prevalence (8–10). The possibility that the plasmid was lost during culture of the strains could not be excluded, however. Virulence genes present on the bacterial chromosome are more stable. However, the 12 strains that were negative for all plasmid genes were strikingly negative for the chromosomal genetic markers previously found to be specific for EHEC O26:H11 (21, 24, 30).

Analysis of the CRISPR loci shows that the CRISPR1 locus of the analyzed O26 strains is formed from a single repertoire of spacers. Globally, the CRISPR1 locus is short, with a limited number of spacers. It is typically shorter than the prototypical O26:H11 stx_1 CRISPR1 locus (from reference strain 11368) and those described by Delannoy et al. (22) and Yin et al. (25). It has already been suggested (31) that CRISPR array length may be inversely proportional to the virulence potential of a strain. Our data seem to corroborate this hypothesis, as the strains belonging to the highly virulent new ST29 clone appear to exhibit a shorter CRISPR1 allele length. However, such an observation is not true for the CRISPR2a allele length, as the CRISPR2a loci from the two groups did not differ significantly in length. The CRISPR2a locus of the 12 ST29 strains that are negative for all plasmid genes has an interesting feature, however, as it contains a large transposon within the first spacer. This transposon is found in CRISPR2 alleles 67 and 71. Allele 67 was previously described in an O26:H11 isolate from cattle carrying a stx_2 gene (25). It was not found previously in human isolates. Allele 71 was observed previously in human *stx*-negative diarrheagenic *E. coli* O26 strains (32). It was not previously found to be related to *stx*-positive strains associated with bloody diarrhea and hemolytic uremic syndrome (HUS). Overall, CRISPR typing appears to be more discriminative than MLST, as a total of 7 CRISPR types (CTs) were identified for only 2 STs. Also, CRISPR typing but not MLST allowed identification of the strains that were negative for all plasmid and virulence genes. Thus, CRISPR typing provided the best strain profiling resolution, corroborating recent findings for other STEC serotypes such as *E. coli* O113:H21 (26). Moreover, our previous studies on *E. coli* O26 showed that O26 CRISPR loci can be used to differentiate clearly between EHEC and non-EHEC strains (22). Interestingly, we also showed in those previous studies that O26 strains with H-types other than H11 (O26:HND, O26:H31, O26:H34, and O26:H32) harbor significantly different CRISPR loci (22).

Although all strains seem to have closely related genetic backgrounds phylogenetically, as ST21 and ST29 differ by one nucleotide in the *adk* locus, their different CRISPR types suggest different evolutionary niches or pathways. CRISPR arrays are thought to act as an adaptive immune system, in which the spacer sequences of the arrays would be derived from foreign DNA following exposure to plasmids or phages (33, 34). Thus, different spacer compositions of CRISPR arrays may indicate exposure to different phage and plasmid environments. Similarly, STEC strains with different niches (i.e., exposed to different spectra of aggressors) will likely have different CRISPR arrays. Accordingly, all ST21 strains and the four ST29 strains (with the plasmid gene profile of *ehxA*+/*katP*−/*espP*−/*etpD*+ and the presence of all virulence genes) (CRISPR types CT1, CT4, CT5, CT6, and CT7) most probably share a background, as they exhibit similar CRISPR arrays. Similarly, the 12 ST29 strains (negative for all plasmid genes) have related CRISPR arrays and probably evolved in a common environment. This hypothesis is corroborated by the presence of the stx_{2d} gene subtype in 7 of the 12 ST29 strains. To our knowledge, this is the first time that *E. coli* O26:H11 strains carrying stx_{2d} have been described in humans. However, one stx_{2d}-positive STEC O26 strain (D618/98) was isolated from cheese and cattle in Germany in 1998 (21, 30). The Stx2d-activatable toxin is usually produced by *eae*-negative STEC strains of serotypes O113:H21 and O91:H21 that carry stx_{2d} alone and that were found to be associated with severe diseases such as bloody diarrhea and HUS (35).

The stx_{2d} gene sequences in all 7 strains are identical and are most similar to reference strain 5905 (*E. coli* O55:H7), which was isolated from food in 1994. *E. coli* O26 is a highly dynamic group capable of acquiring *stx*-encoding phages (7). Although *stx* genes are generally highly conserved and show little sequence variation (29), it is likely that these stx_{2d}-positive strains resulted from a single acquisition. Our findings highlight the continuing evolution of virulent STEC O26 strains and remind us that previously observed stable virulence gene combinations are subject to change (e.g., stx_{2d} is now found in combination with *eae*). It was postulated by Bletz et al. that a diverse population of EHEC O26 strains circulated over a long period of time in an evolutionarily stable niche (source) and only a few strains were able to adapt during the transfer into a new niche (sink), with positive purifying selection (36). Highly pathogenic EHEC O26 strains of the new clones carrying solely stx_{2a} or stx_{2d} have only rarely been isolated outside humans. Further studies are necessary to elucidate potential reservoirs of these new clones and to understand the evolutionary dynamics between source and sink.

In an attempt to improve the current detection scheme for EHEC in food samples, we previously identified genetic markers associated with EHEC O26:H11 (21, 22, 24). Analysis of the plas-

mid and chromosomal virulence gene contents of the strains showed that 12 strains of ST29 were unexpectedly negative for all of the tested genetic markers. In order to detect these particular strains, including the *E. coli* O26:H11 strain carrying the stx_{2d} gene, we developed a new CRISPR-based O26:H11 PCR assay, SP_O26_E. This new CRISPR-based O26:H11 PCR assay may be a useful addition for fully investigating STEC O26:H11 isolates that are associated with severe clinical outcomes, i.e., bloody diarrhea and HUS. A complete evaluation of this PCR assay with a large collection of strains should be performed in order to validate its use in STEC O26:H11 characterization. Further studies in France or in other countries will be necessary to better determine whether O26 strains with these profiles of *stx* types and genotypes are statistically significant in humans.

ACKNOWLEDGMENT

The project was partially financed by the French Joint Ministerial Program of R&D against CBRNE Risks.

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